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Evaluation of RU58841 as an Anti-Androgen in Prostate PC3 Cells and a Topical Anti-Alopecia Agent in the Bald Scalp of Stumptailed Macaques

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The effect of androgen receptor transcriptional activation by RU58841, a nonsteroidal anti-androgen, was studied in the human prostate cancer PC3 cell line by cotransfection with wild-type androgen receptor (wt AR) and an androgen-responsive reporter (MMTV-ARE-CAT) construct. Anti-androgens, hydroxyflutamide, and Casodex, and the antiestrogen, genistein, were studied in parallel for comparison with RU58841. The wt AR was activated only by the androgen dihydrotestosterone (DHT). Neither the anti-androgens nor antiestrogen can enhance AR transcriptional activity at 10^{-11}–10^{-7} *M* in PC3 cells. Hydroxyflutamide, RU58841, and Casodex, but not genistein, displayed competitively suppressive effects on DHT activation of wt AR. The potency of RU58841 was comparable to that of hydroxyflutamide. From this result, topical application of RU58841, which is considered to be a potential therapy for skin diseases, may induce systemic side effects. However, RU58841, on topical application, revealed a potent increase in density, thickening, and length of hair in the macaque model of androgenetic alopecia, whereas no systemic effects were detected. Together our results suggest that RU58841 may have potent antagonism to the wt AR and could be considered as a topically applied active anti-androgen for the treatment of androgen-dependent skin disorders, such as acne, androgenetic alopecia, and hirsutism.

Key Words: Hydroxyflutamide; Casodex; alopecia; prostate.

Introduction

Anti-androgens antagonize the biological responses induced by endogenous or exogenous androgens by competitively inhibiting their binding to the androgen receptor (AR) *(1–3)*. The specificity of androgen action is accomplished both by the specific recognition of target genes and by the specificity of the androgen–AR interaction *(4,5)*. Pure anti-androgens are nonsteroidal compounds that bind exclusively to the AR *(6)*. Hydroxyflutamide, one of the nonsteroidal anti-androgens, is used in the treatment of prostate cancer *(7–9)*. More recently another nonsteroidal compound, Casodex, has been used for the same indication *(10,11)*. The common feature of these anti-androgens is their weak relative binding affinity (RBA) for the AR *(12)*. In contrast, RU58841, a new topically active nonsteroidal anti-androgen, displays a high binding affinity for the AR *(13)*. When topically applied in vivo, it exerts a potent dose-dependent regression of the hamster flank organ *(13)*. Genistein, a plant-derived nonsteroidal isoflavonoid, has been shown to have high binding affinity for the estrogen receptor *(14)* and exert concentration-dependent estrogenic or antiestrogenic properties *(15,16)*. We assessed genistein for possible anti-androgenic effects.

In the present study, we compared the effect of these compounds on the human wild-type (wt) AR. We used PC3 cells, which were derived from metastatic lesions of a human prostatic carcinoma, containing no functional endogenous AR. To study transcriptional activation by the wt AR, we transfected PC3 cells with a wt AR, and an androgen-responsive reporter (mouse mammary tumor virus promoter–androgen-response element–chloramphenical acetyltransferase [MMTV-ARE-CAT]) construct, and then measured CAT activity in the presence of the various anti-androgens. The results showed that the antagonistic effect of RU58841 is as potent as that of hydroxyflutamide. This indicated that RU58841 may induce systemic effects by its topical application, because in the previous report,

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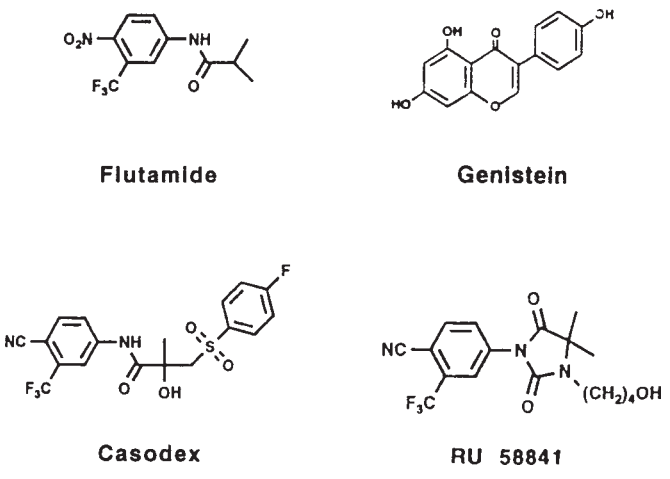


Fig. 1. Chemical structures of anti-androgens flutamide, Casodex, RU58841, and genistein.

the unilateral application of flutamide to flank organs resulted in bilateral reductions, suggesting a systemic mode of action *(17)*. In our in vivo study, however, the topical application of RU58841 (a 5% solution in a propyleneglycol and isopropanol mixture) induced, without any systemic effect, regrowth of thick terminal hairs from vellus hairs in the bald scalp of the stumptailed macaques, which has been used as a model for the study of human androgenetic alopecia *(18,19)*. These data suggest RU58841 could be an important agent for the treatment of androgenetic alopecia and hirsutism, as well as an anti-androgen to be used in prostatic cancer and other androgen-sensitive disease states.

Results and Discussions

The transcriptional activation of the wt AR was determined by CAT activity. The activities of hydroxyflutamide (the active metabolite of flutamide), Casodex, genistein, and RU58841 (*see* structure in Fig. 1) were compared to that of dihydrotestosterone (DHT). As shown in Fig. 2, only DHT exhibits high CAT activity in PC3 cells cotransfected with an androgen-responsive reporter gene and the wt AR. None of the anti-androgens or genistein induced CAT activity at concentrations of 10^{-11}–$10^{-7}M$, indicating that none of these compounds exerted agonistic effects on the wt AR in PC3 cells. Although earlier reports have demonstrated that hydroxyflutamide can also induce some CAT activity when wt AR or mutant AR were present in DU145 cells or COS cells *(20,21)*, this may be due to the presence of AR complexes containing the anti-androgen-AR and specific cofactors in the different cell lines, and interaction between these components may modify the AR's activity on exposure to anti-androgens.

In competition studies, hydroxyflutamide and RU58841 suppressed DHT's effects in PC3 cells with the wt AR in a dose-dependent manner, indicating that these two compounds exert antagonistic effects (Fig. 3A,B). The IC_{50} value for RU58841 was 100 n*M*, which is similar to that of hydroxyflutamide. The potency of RU58841 in this transient transfection study was not as high as expected given its high binding affinity for the AR (k_a = 1.1 n*M*). A newly proposed tripartite system (ligand–receptor–cofactor) may explain this result *(22)*. This system proposes that different cells may have different molecular components, for example, different cofactors, which interact with the agonist–receptor or antagonist–receptor complexes, and subsequently activate or inhibit transcriptional activity. Therefore, the affinity between agonist/antagonist and receptor may not be correlated with the degree of target gene activation/repression. This can also explain why Casodex, an anti-androgen with a nearly threefold greater binding affinity than hydroxyflutamide to the AR *(23)*, was shown to be a less potent anti-androgen than hydroxyflutamide in the DHT-suppression assay (Fig. 3C). Genistein had no potent anti-androgenic effects in our competition study, but displayed a mild androgenic effect at higher concentrations (Fig. 3D).

As shown in Fig. 3, RU58841 has a potent anti-androgenic effect, which is similar to that of hydroxyflutamide. Based on this finding, RU58841 may have not only a topical effect *(13)*, but also a systemic effect in vivo, because topical flutamide has been suggested to induce systemic effects *(17)*. From this point of view, we were interested in evaluating RU58841 as a topical drug, especially for androgenetic alopecia. We applied 5% RU58841 on the bald scalp of the stumptailed macaques. The folliculogram analysis revealed that all four cases treated with 5% RU58841 showed a marked progressive pattern of folliculograms in 5 mo (Fig. 4B). The population of anagen follicles was greatly increased and that of telogen follicles was reduced compared to time zero of treatment (Fig. 4A). Vehicle application did not induce any effect on hair regrowth throughout the 5-mo period of treatment (data not shown). These results demonstrate RU58841-treated cases had a much higher rate of cyclic progression from telogen to anagen follicles and greater enlargement of follicular size compared to those of vehicle-treated cases. On the other hand, examination for possible systemic effects of topical RU58841 in the treatment group showed no detectable abnormalities in body weight, hematology, and blood chemistry tests, serum levels of testosterone, dihydrotestosterone, and luteinizing hormone (data not shown).

A common feature of pure anti-androgens, such as hydroxyflutamide and Casodex, is their relatively weak binding affinity for the AR, 50–100 times less than that of testosterone *(6,23,24)*. In contrast to these two anti-androgens, RU58841 exhibits a high and specific binding for the AR, equivalent to or higher than that of testosterone *(13)*.

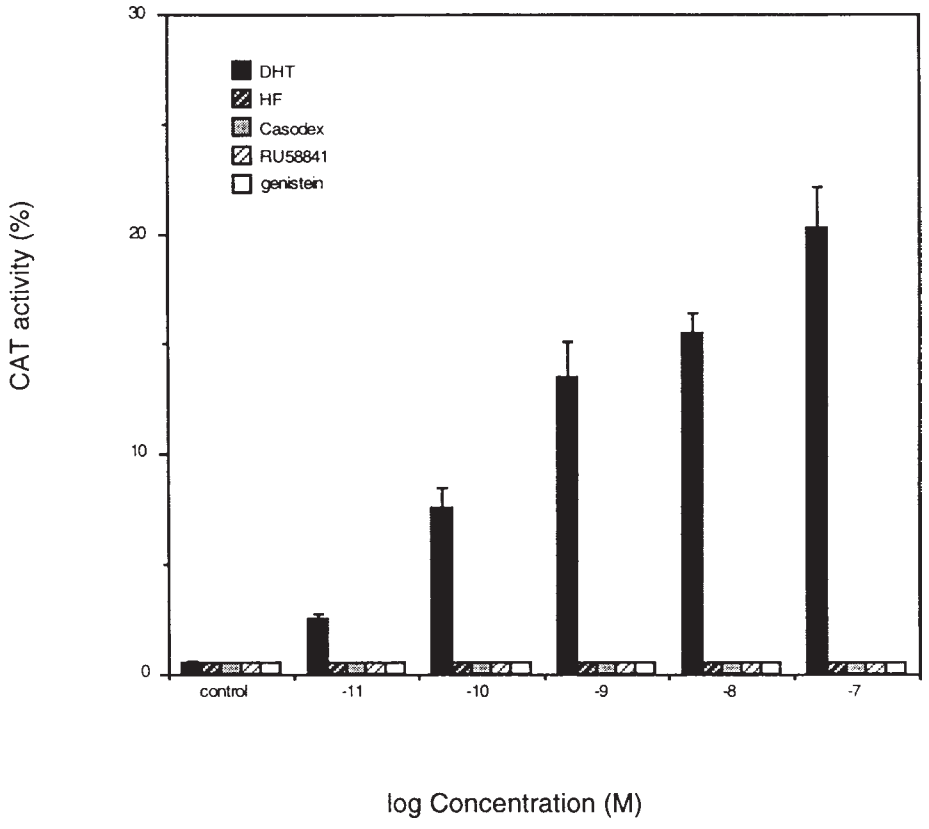


Fig. 2. Induction of CAT activity in PC3 cells cotransfected with wt AR and MMTV-CAT reporter plasmids. Cells were treated with different compounds at various concentrations: DHT, hydroxyflutamide (HF), Casodex, RU58841 and genistein 24 h after transfection. Activity is expressed as CAT conversion rates (%), which were calculated from PhosphorImager (Molecular Dynamics) quantifiable intensities. (C; control incubation, without androgen or anti-androgen.) The data are shown here as mean ± SE of three independent experiments.

Moreover, when topically applied, RU58841 exerts a potent dose-dependent regression of the hamster flank organ *(13)*. Likewise, in our study, topical RU58841 induced remarkable affects on hair and follicular regrowth in the bald frontal scalp of macaques. This observation indicates that topical RU58841 is a clinically hopeful therapy for androgenetic alopecia. Furthermore, the effect of RU58841 on human hair production on balding scalp grafts maintained on testosterone-conditioned nude mice has been demonstrated *(25)*. This is contrary to cyproterone acetate, which is active in the hamster flank organ model, but inactive in human when applied topically *(26)*. In addition to in vivo topical effects, RU58841 also antagonized testosterone-elicited follicular cell growth inhibition in vitro *(27)*.

Anti-androgens are potential drugs for androgenetic alopecia, because androgens have been shown to be key molecules for pathogenesis of androgenetic alopecia *(27)*. Although topical application of anti-androgens can be a hopeful therapy, systemic side effects, such as impotentance and gynecomastia, must be ruled out. In our trial of RU58841 for androgenetic alopecia of macaques, it displayed specific local activity and dissociation between local and system effects. These results indicate that RU58841 may become a promising topically active anti-androgen for the treatment of androgen-dependent skin disorders, such as acne, androgenetic alopecia, and hirsutism.

Materials and Methods

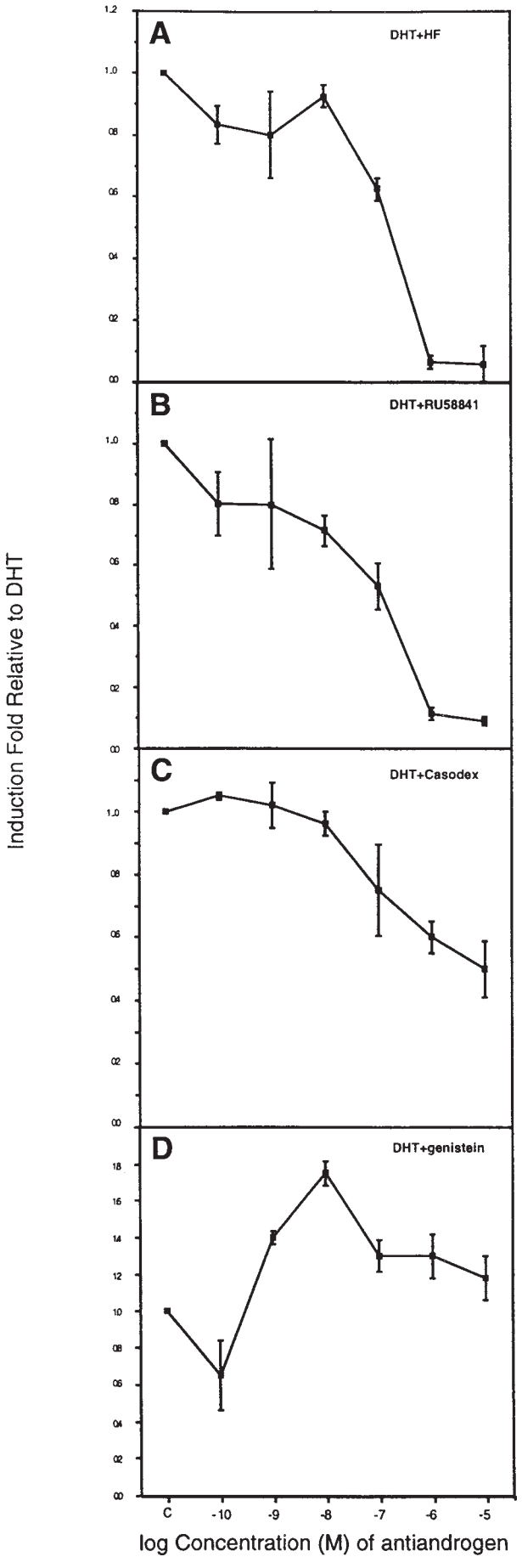
Materials

DHT was purchased from Sigma (St. Louis, MO), hydroxyflutamide from Schering (Bloomfield, NJ), Casodex (ICI 176,334) from ICI Pharmaceuticals (Cheshire, England), RU58841 from Roussel UCLAF (Romainville, France), Genistein from Gibco BRL (Gaithersburg, MD), and [^{14}C]chloramphenical was obtained from Amersham (Arlington Heights, IL).

Transient Transfections

Construction of expression vectors, PSG5-AR (PSG5 with wt AR) and culture of PC3 cells were as described previously *(28,29)*.

PC3 cells were routinely maintained in Delbecco's Modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum. Cells (3×10^5) were seeded in 60-mm culture dishes 24 h before transfection.



The media were changed to DMEM with 5% charcoal dextran-stripped serum (CDFBS) at least 1 h before transfection. The cells were transfected by using a modified calcium-phosphate precipitation method with 4 μg PSG5-AR and 4 μg MMTV-ARE-CAT reporter gene. Backbone plasmid (PSG5) was added to a total of 10 μg plasmids/dish. Twenty-four hours after transfection, CDFBS containing different test compounds at the indicated concentrations (Figs. 2 and 3) was added, and culture was continued for another 24 h.

CAT Assays

Twenty-four hours after being cultured in experimental media, the cells were harvested for chloramphenical acetyltransferase (CAT) assay, performed as described previously *(30)*. To normalize the transfection efficiency, a γ-galactosidase expression vector was cotransfected. All experiments were performed in triplicate.

Animal Subjects

Subject animals were male and female stumptailed macaques *(Macaca arctoides)*, in the postpubertal stage (5- to 15-yr old). Each animal was housed in our animal care facility (accredited by the American Association for Accreditation of Laboratory Animal Care to University of Wisconsin Research Animal Resource Center). The animal care protocol number is A-34-8800-G00267.

Topical Application

We applied 0.5 mL 5% RU58841, dissolved in vehicle solution (50% propylene glycol, 30% isopropyl alcohol, 2% isopropyl myristate, and 18% distilled water), to the frontal bald scalp of four adult stumptailed macaques *(M. arctoides)* once per day, 5 d/wk.

Micromorphometry (Folliculogram)

A skin biopsy (4-mm punch) was taken from the frontal scalp of macaques under anesthesia. Serial paraffin sections were cut from the specimens. After staining with hematoxylin and eosin, all hair follicles appearing in the serial sections were traced and their outline drawn using a projecting microscope. After defining their follicular cyclic phases (telogen, mid- and late anagen, and catagen), the length of each follicle was measured. The histograms representing the proportional population of each cyclic phase and follicular size were made to analyze the sequential changes of the follicular enlargement and cyclic progression.

Fig. 3. *(opposite)* Effects of anti-androgens on DHT-induced AR transcription. PC3 cells were cotransfected with a wt AR expression plasmid and a MMTV-CAT reporter plasmid, and incubated with both 1 n*M* DHT and a series of concentrations of different compounds: **(A)** HF; **(B)** RU58841; **(C)** Casodex; **(D)** genistein. CAT activity is expressed in folds of the activity found when treated with 1 n*M* DHT alone (C, control incubation).

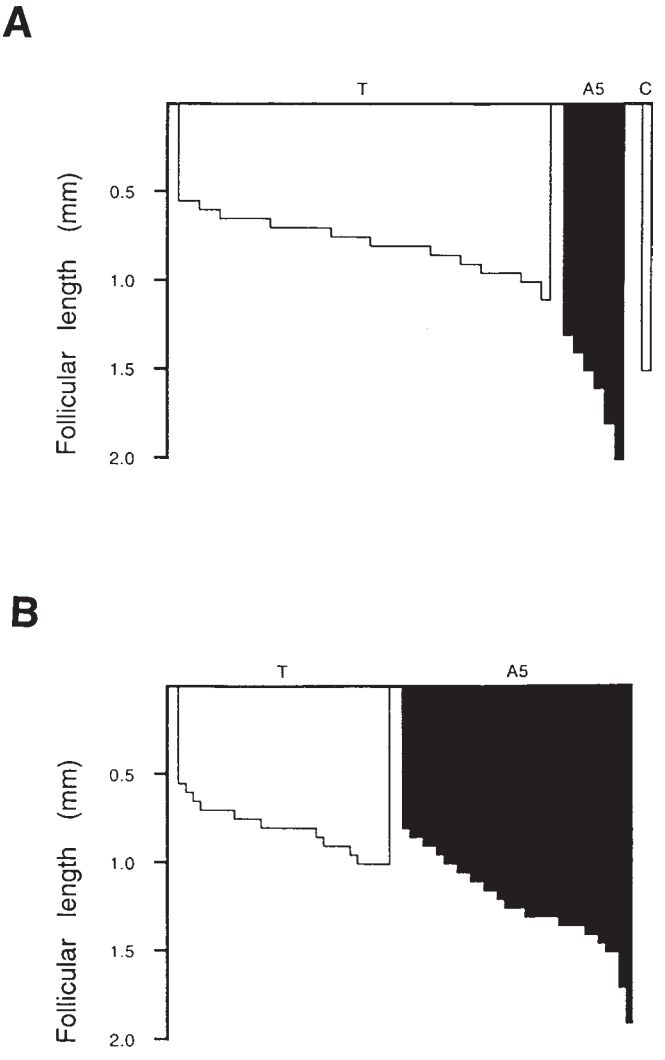


Fig. 4. Folliculogram. Sequential patterns of folliculograms at pretreatment: **(A)** 0 time; **(B)** 5 mo after RU58841, showing increased population, and overall size of anagen follicles (black bar) at 5 mo compared to 0 time. T—telogen, A5—late anagen, C—catagen phase.

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