0026-895X/95/051080-09$3.00/0

MOLECULAR PHARMACOLOGY, 47:1080–1088 (1995).

Tissue-Specific Pharmacology of Testosterone and 5α-Dihydrotestosterone Analogues: Characterization of a Novel Canine Liver Androgen-Binding Protein

ANN E. SUMMERFIELD, PABLO J. DIAZ CRUZ, MICHAEL P. DOLENGA, HOWARD E. SMITH, CATHERINE D. STRADER, and JEFFREY H. TONEY

Department of Molecular Pharmacology and Biochemistry, Merck Research Laboratories, Rahway, New Jersey 07065-0900 (A.E.S., M.P.D., C.D.S., J.H.T.), and Department of Chemistry, Vanderbilt University, Nashville, Tennessee 37232 (P.J.D.C., H.E.S.)

Received June 9, 1994; Accepted March 1, 1995

SUMMARY

The mechanism by which the hormones 5α-dihydrotestosterone and testosterone differentially regulate such diverse functions as development of male internal and external genitalia and maintenance of prostatic growth via a single androgen receptor (AR) is not well understood. To search for potential AR isoforms, an extensive pharmacological survey of the binding of [^{3}H]mibolerone (7α,17α-[^{3}H]dimethyl-19-nortestosterone) in dog prostate, adrenal gland, testis, liver, kidney, brain, muscle, and spleen cytosolic extracts was carried out. The antagonist androst-4-en-3,17-dione (ATD), as well as a series of unsaturated analogues of testosterone, exhibited marked tissue specificity for binding to mibolerone-binding proteins (MBPs), with ATD having a 10-fold higher affinity for the MBPs present in liver than for those in prostate and testis. The difference in affinity was not due to tissue-specific metabolism of ATD. Competition binding profiles for ATD with mixtures of prostate and liver extracts were consistent with two distinct populations of binding sites. Both wild-type human AR-B and the recently discovered human AR-A isoform were expressed in COS cells and were found to exhibit pharmacology similar to that of the prostatic MBPs in dogs. Analogues of ATD or testosterone could prove to be useful probes for delineating the differential effects of 5α-dihydrotestosterone and testosterone on the biological actions of the AR and related proteins.

The AR plays a major role in sexual development and physiology via two hormonal signals, DHT and testosterone (1). DHT affects the development of male external genitalia, whereas testosterone is responsible for virilization of the male internal genital tract. A major unanswered question is the mechanism by which the AR can regulate such diverse functions. Models including one receptor with two ligands versus two receptors and two ligands have been debated (2, 3). Genetic evidence strongly suggests that the human AR is encoded by a single gene. However, there is accumulating evidence that the AR exists as multiple isoforms (4–6) and that effects of DHT and testosterone can be separated pharmacologically (7). Splicing variants of the AR within the 5′ untranslated region have been identified in human LNCaP prostatic carcinoma cells (8) and in rat brain tissue (9). Two mRNA isoforms of the AR have recently been identified in the developing larnyx of male *Xenopus laevis* (10). The AR mRNA α and β isoforms differ within the A/B or hypervariable domain of the receptor, which is believed to be involved in such functions as transcriptional activation, translocation to the nucleus, receptor dimerization, and specificity for hormone response elements (10). More recently, two isoforms of the AR have been described in a single human pedigree in which one form is truncated at the amino terminus (11), analogously to the A-form of the progesterone receptor (12). Other members of the steroid/thyroid/retinoic acid superfamily have been found to exist as multiple forms, including the estrogen receptor (13, 14) and the retinoic acid receptor, for which the subtypes can be distinguised pharmacologically (15).

In the present study, we have used a series of androgenic steroids as probes to address the possibility of tissue-specific isoforms of the AR or other ABPs present in a variety of dog tissues. Beagle dogs have been used as an animal model for benign prostatic hyperplasia, a disease that is androgen dependent (16, 17). It is thus considered of interest to identify potential isoforms of the AR and/or ABPs in this species. L-598,229 (5α-20-spiroxan-3-one) has been postulated to act as a DHT-specific antagonist,[1] whereas ATD is hypothesized to act as a testosterone antagonist (7). A series of unsatur-

[1] G. E. Arth, unpublished observations.

ABBREVIATIONS: AR, androgen receptor(s); DHT, 5α-dihydrotestosterone; ABP, androgen-binding protein; ATD, androst-4-en-3,17-dione; TAC, triamcinolone acetonide; DCC, dextran-coated charcoal; HPLC, high performance liquid chromatography; MBP, mibolerone-binding protein; SHBG, steroid hormone-binding globulin.

ated analogues of androst-4-en-17β-ol-3-one were also used as potential ligands for AR/ABP isoforms in dog tissues, because this family of compounds has been shown to bind to rat epididymal ABPs (18). The probe used was [^{3}H]mibolerone (7α,17α-[^{3}H]dimethyl-19-nortestosterone), a nonmetabolizable androgen that is highly selective for the AR in the presence of other steroid/thyroid hormone receptors (19–21). If there exists a single AR/MBP with a distinct pharmacological profile, then the binding affinity of the ligands used would be expected to be the same in all tissues studied, assuming that tissue-specific metabolism of the steroids does not occur under the conditions of the assay. Such a result would suggest that DHT and testosterone interact with similarly modified/expressed receptors. However, the present study demonstrates that these ligands exhibit strikingly different affinities among the tissues studied.

Because human AR-A and AR-B are the only known isoforms of this receptor, we considered it to be of interest to explore the pharmacology of these proteins. To this end, human AR-A and -B were expressed in COS cells, to determine whether the unique phamacology observed in dog tissues could be due to differential expression of these isoforms.

Experimental Procedures

Materials. [^{3}H]Mibolerone (85.3 Ci/mmol), [1,2,4,5,6,7-^{3}H]DHT (198.8 Ci/mmol), [1,2,6,7-^{3}H]testosterone (93.0 Ci/mmol), [1β-^{3}H]ATD (24.1 Ci/mmol), and unlabeled mibolerone were purchased from DuPont-NEN Research Products. Unlabeled TAC, ATD, and estradiol were purchased from Sigma Chemical Co. (St. Louis, MO); testosterone and DHT were from Aldrich Chemical Co. (Milwaukee, WI). ATD and L-598,229 were obtained from Merck and Co. (Rahway, NJ). 17α-Ethynylandrost-4-en-17β-ol-3-one (ethisterone) (**2b**), 17α-[(*Z*)-2-iodoethenyl]androst-4-en-17β-ol-3-one (**2e**), Δ^6-testosterone (**3a**), 17α-ethynylandrost-4,6-dien-17β-ol-3-one (**3b**), 17α-[(*E*)-2-iodoethenyl]androst-4,6-dien-17β-ol-3-one (**3f**), $\Delta^{1,6}$-testosterone (**4a**), and 17α-ethynylandrost-1,4,6-trien-17β-ol-3-one (**4b**) were synthesized and purified as described (18). Activated charcoal was purchased from EM Scientific (Gibbstown, NJ), dextran T-70 from Pharmacia (Uppsala, Sweden), and gelatin (type IV, from calf skin) from Sigma. All other chemicals were of reagent grade and were purchased from Sigma.

Preparation of tissue cytosols. Unless otherwise specified, all tissues were obtained from young (9–12-month old) beagle dogs. Pharmacological studies carried out using livers from male and female dogs yielded the same results, within experimental error. All other studies were carried out using tissues obtained from male dogs. All animal procedures were performed in accordance with the highest standards for the humane handling, care, and treatment of research animals and were approved by the Merck Institutional Animal Care and Use Committee. The care and use of research animals at Merck meets or exceeds all applicable local, national, and international laws and regulations. Tissues were removed immediately after sacrifice and frozen in liquid nitrogen until use. All further procedures were carried out at 0–4° unless otherwise specified. Frozen tissue was pulverized at liquid nitrogen temperatures in a SPEX 6700 Freezer Mill and stored as a powder at −80°. For each experiment, an appropriate volume of tissue powder was added to 2–3 volumes of fresh TEGM buffer (10 mM Tris·HCl, 1 mM EDTA, 10%, v/v, glycerol, 1 mM β-mercaptoethanol, 10 mM sodium molybdate, pH 7.2, containing 2.5 mM leupeptin, 0.1 mM bacitracin, 1.0 mM phenylmethylsulfonyl fluoride, and aprotinin at 200 units/100 ml of buffer) and then homogenized at 0° using a Brinkmann Polytron PT3000 homogenizer (Kinematica AG). Homogenization was performed in three 25-sec periods separated by 30-sec intervals of cooling, followed by three strokes using a Dounce homogenizer. The homogenate was then centrifuged at 100,000 × *g* for 60 min. Total protein levels in cytosolic samples were determined using the method of Bradford (22).

Radioligand binding assays. Aliquots of tissue cytosol were added to prechilled borosilicate glass tubes containing variable amounts of ^{3}H-labeled ligand (to determine total binding) or variable amounts of ^{3}H-labeled ligand and an excess of unlabeled ligand (to determine nonspecific binding). Labeled and unlabeled ligands were added to each assay tube as a 100-fold concentrated ethanol stock solution. Unless otherwise noted, the assay volume was 500 μl. All samples contained TAC at a concentration 1000-fold higher than that of the radioactive ligand, to block binding to competing steroid hormone receptors (e.g., glucocorticoid and progesterone receptors). All binding reactions, performed in duplicate, were incubated overnight (18–20 hr) at 4° and then terminated by addition of an excess (40% of the assay volume) of DCC (5%, w/v, charcoal, 0.5%, w/v, dextran T-70, and 0.1%, w/v, gelatin in Tris-EDTA buffer, pH 7.2) chilled to 4°. DCC was added to each binding reaction to separate protein-bound and free steroids. Treatment with DCC was performed at 4° for roughly 3–5 min, followed by centrifugation at ~3000 × *g* for 20 min. An aliquot of the supernatant was removed and the radioactivity was determined by scintillation counting. After the measurement of radioactivity, binding parameters such as ligand dissociation constant (K_d) and maximum number of binding sites per milligram of total protein (B_{max}) were calculated according to the method of Scatchard (23), using data corrected for nonspecific binding, which was determined in the presence of a 1000-fold excess of radiolabeled ligand. Specific binding was calculated as the difference between total and nonspecific binding. The data were fit using a standard linear least-squares algorithm (KaleidaGraph data analysis/graphics software; Synergy Software, Reading, PA).

Ligand specificity studies. Aliquots of cytosol were added to prechilled borosilicate glass tubes containing [^{3}H]mibolerone at a final concentration of 0.1 nM (approximately the K_d value), unlabeled TAC at a final concentration of 100 nM, and an appropriate amount of unlabeled competitor as stated in the figure legends. Total binding in the absence of competitor was determined from incubations of cytosol with [^{3}H]mibolerone and TAC. Nonspecific binding was determined from incubations containing 100 nM unlabeled mibolerone in addition to [^{3}H]mibolerone and TAC and was typically ≤10–25% of total binding. All incubations were performed as described above. Competition curves for each competitor compound were generated using data that had been corrected for nonspecific binding. IC_{50} values were calculated using an RS1 implementation of the National Institutes of Health AllFit program. K_d values for ligand competition data were calculated according to the Marquardt algorithm (24).

Measurement of metabolism of steroids in cytosol. Mibolerone, testosterone, DHT, TAC, and ATD were isolated using reverse phase HPLC. Tissue cytosols containing these compounds (ATD at a final concentration of 25 μM, DHT at 100 μM, and all other compounds at 50 μM) were first extracted three times with HPLC-grade ethyl acetate (using a 1:1 cytosol/ethyl acetate volume ratio). After each extraction, cold ethyl acetate was added to the cytosol, and the sample was vortex-mixed and incubated on ice for 15 min. The sample was briefly vortex-mixed again and then centrifuged at 4000 rpm at 4° for 10 min in a Hermle Z360K benchtop centrifuge. The three ethyl acetate extracts were combined and placed in a Speed Vac concentrator to evaporate the samples to dryness. The remaining residue was redissolved in HPLC mobile phase (15% acetonitrile/42.5% methanol/42.5% water) and filtered through a 0.22-μm filter. Steroids were then resolved using an isocratic reverse phase HPLC system, as described (25). Determination of the extent of steroid metabolism was made by comparing the peak area of each compound extracted from incubated cytosol with the peak area of the same compound extracted either from a control buffer solution or from a control cytosol solution.

Aromatase assays. A tritium water release assay was employed to measure the activity of aromatase, using [1β-^{3}H]ATD as a sub-

strate (26). Cytosolic extracts prepared as described above were diluted into TEGM buffer to final protein concentrations similar to those used in the AR assays. Assays carried out under optimal conditions used an assay premixture containing cofactors required for aromatase activity. The assay premixture contained the following components dissolved in buffer D (50 mM KH_2PO_4, 0.5 mM EDTA, 5 mM $MgCl_2$, 0.5 mM 1,4-dithiothreitol): 250 nM [^{3}H]ATD, 250 nM unlabeled ATD, 2.5 mM glucose-6-phosphate, 0.25 units/ml glucose-6-phosphate dehydrogenase, and 0.25 mM NADPH. Both [^{3}H]ATD and unlabeled ATD were dissolved in ethanol, taken to dryness, and resuspended in 1,2-propanediol at <1% of the total assay premixture volume. Competitor compounds dissolved in ethanol to the appropriate concentration were transferred into borosilicate glass test tubes in triplicate and taken to dryness. Assay premixture (0.25 ml) was then added to each tube, followed by 0.20 ml of cytosolic extract, and the contents were mixed. Reactions were carried out at 30° for 30 min and then terminated by the addition of 0.25 ml of prechilled trichloroacetic acid (20%, w/v). Free [^{3}H]ATD was then extracted using 2 ml of chloroform, by vigorous vortex-mixing for 30 sec. The samples were then centrifuged at ~300 × *g* for 3 min, and 0.4 ml of the supernatant was transferred to fresh test tubes. Residual [^{3}H]ATD was then removed using 0.4 ml of DCC as described above. Each supernatant (0.5 ml) was then transferred to a scintillation vial and the radioactivity was counted. Reaction controls contained all components in the buffer except NADPH. To allow direct comparison with the AR assay, aromatase activity was also measured using 250 nM [^{3}H]ATD and 250 nM unlabeled ATD in TEGM buffer in the absence of the other components of the assay premixture. Incubations were carried out overnight at 4°. The aromatase activity measured under these conditions represented ≤3% of the activity measured under optimal conditions.

Amplification of AR cDNAs. Total RNA was isolated from beagle dog prostate, liver, and kidney and reverse transcribed into cDNA. The cDNA was amplified using the polymerase chain reaction with primers hybridizing at nucleotides 2413 and 2860, to encompass the ligand binding domain of the human AR (11), as well as several other positions within the possible eight intron/exon boundaries. Amplifications were also carried out using poly(A)$^+$ RNA from human liver, kidney, and testis (Clontech). The resultant products of expected size were then subcloned into a TA vector (Invitrogen), and the DNA was sequenced using the PRISM Ready Reaction DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems).

Cloning and expression of human AR-A and -B in COS cells. Wild-type human AR-B inserted into expression vector CMV-2 (kindly provided by Prof. M. McPhaul, Southwestern Medical Center, Dallas, TX) was digested using *Sma*I to cleave the cDNA between Met-1 and Met-188 (11). The generated DNA fragment was gel purified and inserted into the *Eco*RV site of expression vector pcDNA3 (Invitrogen). The desired human AR-A clone was isolated using colony hybridization techniques and confirmed by DNA sequence analysis of approximately 400 nucleotides from each end of the cDNA. Transfections were carried out using COS-1 cells, by electroporation (Bio-Rad Gene Pulser). Cells were resuspended at 10^7 cells/ml in phosphate-buffered saline containing either 50 μg of CMV3.1 (wild-type human AR-B), human AR-A, or pcDNA3 as a vector control. Electroporation was accomplished using a single pulse (~13 sec) at 260 V and 960 μF. After culturing for 2–3 days, cells were harvested by trypsinization and rinsed twice using cold TEGM buffer. Cells were lysed by freeze/thawing and cytosol was prepared as described above. Binding studies were carried out as described above, except that the sample volume was 100 μl and 0.5 nM [^{3}H]mibolerone was used instead of 0.10 nM, to compensate for lower concentrations of protein in these experiments, relative to tissue cytosols. Transfections using pcDNA3 control showed no specific binding above background levels.

Results

[^{3}H]Mibolerone binding to individual tissue cytosols. Binding of [^{3}H]mibolerone, an androgen analogue highly specific for the AR (19–21) (Fig. 1), was measured for cytosolic extracts prepared from dog prostate, adrenal gland, testis, liver, kidney, brain, muscle, and spleen. The optimal amount of cytosol for each assay was determined by measuring the specific binding of [^{3}H]mibolerone as a function of cytosolic protein concentration for each tissue. The highest protein concentration that provided a linear response in specific ligand binding was chosen for use in saturation binding studies and for determination of binding parameters using Scatchard analysis. Fig. 2A shows the saturation binding curve for [^{3}H]mibolerone binding to dog prostate cytosol. The binding data were analyzed using the method of Scatchard to determine the K_d and B_{max} values. The linearity of the resultant Scatchard plot (Fig. 2B) indicates that, under the conditions of the assay, [^{3}H]mibolerone binds to a single class of sites in the dog prostate, with an average K_d value of 0.20 ± 0.12 nM and an average B_{max} value of 37 ± 9 fmol/mg (five experiments).

Saturation curves for [^{3}H]mibolerone binding to adrenal gland, liver, kidney, and testis cytosols resembled that obtained using prostate cytosol, suggesting the existence of a single class of high affinity mibolerone binding sites. A Scatchard plot generated from the liver cytosol data shown in Fig. 2C is presented in Fig. 2D. In the case of testis, specific binding of mibolerone could be measured only if endogenous steroids were removed from the cytosol, using DCC, before the incubations with [^{3}H]mibolerone were carried out. Treatment of cytosols from other tissues, including prostate, liver, adrenal gland, and kidney, using DCC did not significantly alter the Scatchard parameters or the IC_{50} values measured in ligand specificity studies (see below). K_d and B_{max} values calculated for [^{3}H]mibolerone binding to each tissue cytosol, as well as to expressed human AR-A and -B, are presented in Table 1. [^{3}H]Mibolerone binding to dog muscle and brain cytosols was very low and could not be used to generate reliable binding curves, due to high nonspecific binding. In addition, [^{3}H]mibolerone binding to dog spleen cytosol was measured as a negative control. No binding above background levels was detected, consistent with the observation that the AR is absent from this tissue in male rats (27).

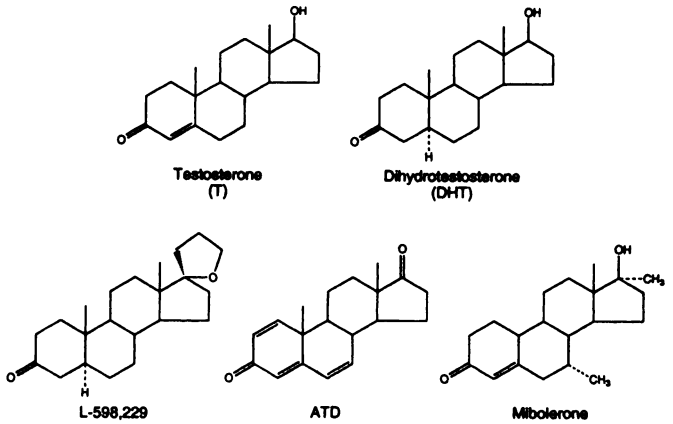


Fig. 1. Chemical structures of androgens and androgen analogues used in radioligand binding experiments.

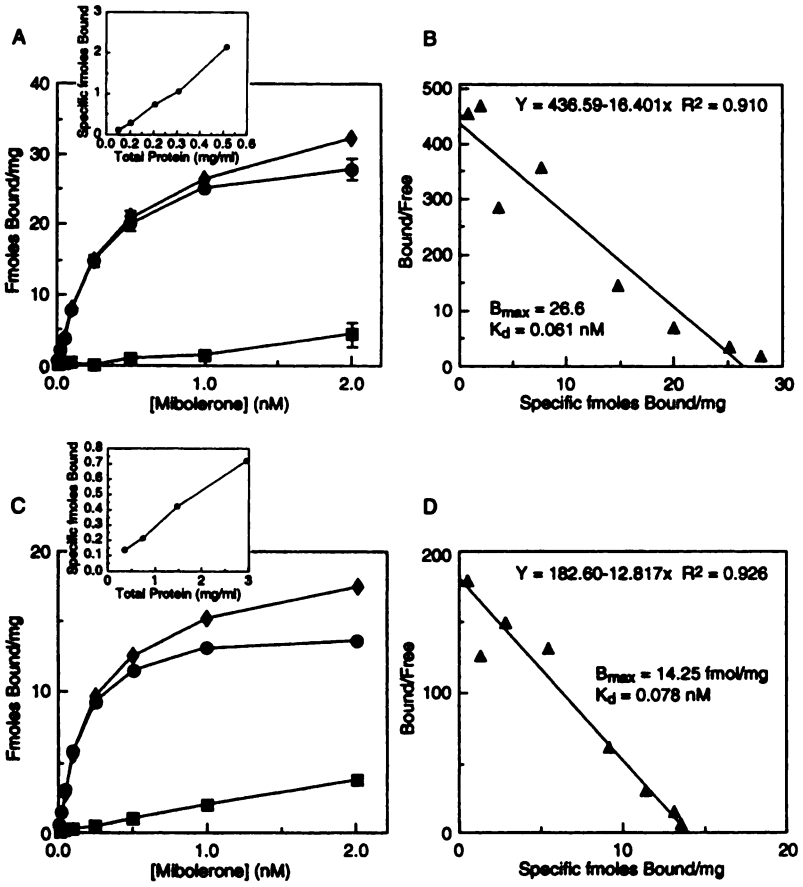


Fig. 2. Binding of [^{3}H]mibolerone to dog prostate (A and B) and liver (C and D) cytosol. A and C, Total (♦), nonspecific (■), and specific (●) binding of [^{3}H]mibolerone. *Insets*, dependence of [^{3}H]mibolerone specific binding on protein concentration. B and D, Scatchard plots of data presented in A and C, respectively. Data shown are taken from a representative experiment. The protein concentration of prostate cytosol was 0.73 mg/ml and that of liver cytosol was 3.4 mg/ml.

TABLE 1

K_d and B_{max} values for [^{3}H]mibolerone binding to dog tissue cytosols and to expressed human AR

Values shown are mean ± 1 SD.

	n^a	K_d	B_{max}
		nM	*fmol/mg*
Tissue			
Prostate	5	0.20 ± 0.12	37 ± 9
Liver	6	0.10 ± 0.02	14 ± 5
Kidney	3	0.05 ± 0.03	2.3 ± 0.6
Adrenal gland	2	0.10 ± 0.03	30 ± 15
Testes	2	0.18 ± 0.05	12 ± 1
Expressed human AR			
Wild-type human AR-B	2	0.23 ± 0.10	1200 ± 200
Human AR-A	2	0.26 ± 0.14	470 ± 300

[a] *n*, number of independent experiments.

Binding by other ^{3}H-labeled ligands. Binding of [^{3}H]DHT and [^{3}H]testosterone was measured for dog prostate cytosols. Scatchard analysis for both ligands yielded linear plots, indicating the existence of a single high affinity binding site for each ligand, with K_d values of 0.26 ± 0.05 nM (two experiments) for DHT and 0.73 ± 0.10 nM (three experiments) for testosterone. The numbers of [^{3}H]DHT and [^{3}H]testosterone sites (B_{max}) measured for prostate cytosol were similar for each ligand (27.9 ± 0.1 fmol/mg for [^{3}H]DHT, two experiments, and 32 ± 9 fmol/mg for [^{3}H]testosterone, three experiments) and were within experimental error of the value calculated for [^{3}H]mibolerone binding to this tissue cytosol (37 ± 9 fmol/mg).

Binding of [^{3}H]testosterone was measured for kidney cytosol as well (data not shown). A K_d value of 0.76 nM and a B_{max} value of 2.7 fmol/mg were calculated from the linear Scatchard plot (one experiment). This K_d value is similar to that obtained for [^{3}H]testosterone binding in prostate tissue and indicates that the AR/MBPs in the two tissue cytosols have similar affinities for testosterone. The B_{max} calculated for kidney cytosol also agrees well with that obtained for [^{3}H]mibolerone binding to the same cytosol (2.3 ± 0.6 fmol/mg). Attempts to measure binding of [^{3}H]testosterone or [^{3}H]DHT using liver or adrenal gland cytosols did not yield values above background levels, due to high nonspecific binding.

Ligand specificity studies for tissue cytosols. The ability of each of the unlabeled compounds L-598,229, ATD, DHT, testosterone, estradiol, and mibolerone to compete with [^{3}H]mibolerone binding to the AR/MBPs was measured for the tissue cytosols examined previously, including prostate, testis, adrenal gland, liver, and kidney. L-598,229 was chosen as a DHT-specific antagonist and ATD was chosen as a testosterone-specific antagonist. Table 2 presents the calculated IC_{50} values for each competitor compound. Fig. 3 displays the competition curves for each of these unlabeled compounds obtained with prostate cytosol. Each of the curves had a Hill coefficient close to 1.0, suggesting a single class of binding sites. The IC_{50} value for testosterone averaged over four experiments was approximately 3–5-fold higher than that for DHT, consistent with the results of [^{3}H]testosterone and [^{3}H]DHT binding. L-598,229 competed for [^{3}H]mibolerone binding but with about 15 times lower affinity, compared with the endogenous ligand DHT. ATD also competed for [^{3}H]mibolerone binding, but very weakly (IC_{50} = 1.8 μM).

TABLE 2

IC_{50} values for compounds competing with [^{3}H]mibolerone for binding in dog tissue cytosols versus expressed human AR

All values are mean ± 1 SD.

	n[a]	IC_{50}					
		L-598,229	ATD	DHT	Testosterone	Estradiol	Mibolerone
		nM					
Tissue cytosol (0.10 nM [^{3}H]mibolerone)							
Prostate	4	3.4 ± 2.0	1800 ± 270	0.22 ± 0.17	1.1 ± 0.40	12.0 ± 0.25	0.29 ± 0.20
Testes	2	6.8 ± 3.5	3300 ± 1300	0.30 ± 0.16	0.8 ± 0.6	8.8 ± 3.6	0.36 ± 0.22
Adrenal gland	3	7.0 ± 3.7	2000 ± 1400	0.7 ± 0.4	0.8 ± 0.6	5.3 ± 2.3	0.33 ± 0.22
Liver	4	71 ± 15	160 ± 32	5.0 ± 2.9	10.7 ± 6.9	5.9 ± 0.5	0.25 ± 0.11
Kidney	3	1.6 ± 0.3	350 ± 120	0.47 ± 0.22	1.1 ± 0.9	6.6 ± 3.5	0.20 ± 0.10
Expressed human AR (0.50 nM [^{3}H]mibolerone)							
Wild-type human AR-B	2	ND[b]	7000 ± 1000	1.10 ± 0.11	2.6 ± 0.60	ND	1.11 ± 0.11
Human AR-A	2	ND	5400 ± 800	0.58 ± 0.08	2.1 ± 0.74	ND	0.79 ± 0.24

[a] *n*, number of independent experiments.
[b] ND, not determined.

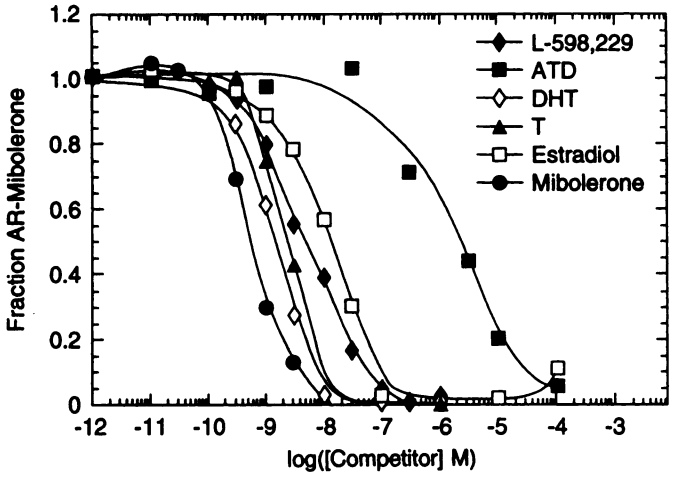


Fig. 3. Competition by various androgen analogues for [^{3}H]mibolerone binding to the AR present in dog prostate cytosol. All samples contained [^{3}H]mibolerone at 0.1 nM and TAC at 100 nM. Prostate cytosol was diluted to ~0.6 mg/ml. Data are smoothed as a guide to the eye and are representative of four independent experiments. *T*, testosterone.

A similar pharmacological profile was observed using the human AR-A and -B isoforms expressed in COS cells (Table 2), confirming that the pharmacology of the AR/MBPs present in dog prostate is the same as that of the AR/MBPs present in human prostate.

Competition curves generated for adrenal gland, liver, kidney, and testis were found to differ from those obtained for prostate. For each cytosol, competition curves were roughly parallel to one another and were best described by a single class of binding sites. The IC_{50} values for mibolerone in all five tissue extracts tested ranged between 0.20 nM and 0.36 nM, consistent with the results of [^{3}H]mibolerone binding. The IC_{50} value for estradiol was relatively constant, at 5–12 nM, for all tissue cytosols. IC_{50} values for DHT and testosterone were also consistent among the tissue extracts studied, with the exception of liver, in which the IC_{50} values were found to be approximately 10-fold higher, i.e., 5.0 ± 2.9 nM (DHT) and 10.7 ± 6.9 nM (testosterone). L-598,229 was a relatively high affinity competitor of [^{3}H]mibolerone binding to prostate, adrenal gland, testis, and kidney, with IC_{50} values ranging from 1.6 to 7.0 nM. In contrast, L-598,229 was a weaker competitor of [^{3}H]mibolerone binding to liver, with an IC_{50} value of approximately 71 nM. The antagonist ATD was a weak competitor of [^{3}H]mibolerone binding to prostate, testis, and adrenal gland cytosols, with IC_{50} values of approximately 2–3 μM, but a much stronger competitor in kidney and liver cytosols, with IC_{50} values of 350 nM and 160 nM, respectively.

Because of the surprising result of higher affinity binding of ATD to the AR/MBPs present in liver, compared with prostate, we analyzed structural analogues to determine whether a family of compounds might exist that also exhibit such tissue-specific binding. The ability of a variety of these analogues to compete with [^{3}H]mibolerone binding to the AR/MBPs in both prostate and liver cytosols was tested. As presented in Table 3, several of the compounds within a series of unsaturated analogues of testosterone exhibited stronger binding to AR/MBPs in liver, relative to those in prostate, including **3a** (Δ^6-testosterone), **4a** ($\Delta^{1,6}$-testosterone), **2b**, **3b**, and **4b**. In contrast, the iodoethynyl derivate **2e** exhibited stronger binding to the AR/MBPs present in prostate, as did testosterone itself. Because **3a** exhibited much higher affinity for the AR/MBPs present in dog liver, relative to prostate, this compound was also studied using human AR-A and -B expressed in COS cells. The affinity of **3a** for both human AR-A and -B was similar to that observed for dog prostate AR/MBPs (Table 3).

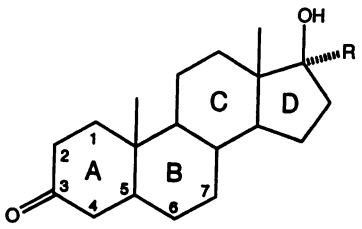
One explanation for the differences in the IC_{50} values for L-598,229 and ATD among the tissues surveyed could be tissue-specific metabolism of compounds during the incubation period of the assay. To address this issue, two series of experiments were performed. In the first series, liver and prostate cytosols were combined in a ratio such that 50% of the [^{3}H]mibolerone binding sites were derived from liver and 50% from prostate. This cytosolic mixture was then used to test the ability of unlabeled L-598,229, ATD, and mibolerone to compete with [^{3}H]mibolerone for binding to the AR. Any tissue-specific metabolic enzymes should be present in the mixture, yielding a single class of binding sites for the metabolized compound.

The results of these experiments using ATD indicated that this compound bound to mixtures of liver and prostate cytosol with an IC_{50} of 420 nM (one-site fit), intermediate between those determined for liver and prostate alone. The ATD competition data for this prostate/liver mixture were best fit to a theoretical curve that assumed two classes of binding sites

TABLE 3

Tissue specificity of unsaturated analogues of testosterone for competing with [^{3}H]mibolerone

All values represent the mean ± standard deviation from two or more independent experiments.



Compound	R	LC_{50}							
		2 (Δ^4)[a]		3 ($\Delta^{4,6}$)				4 ($\Delta^{1,4,6}$)	
		Prostate	Liver	Prostate	Liver	Human AR-A	Human AR-B	Prostate	Liver
						nM			
a	H	1.1 ± 0.4	11 ± 7	17 ± 2	0.08 ± 0.03	22 ± 5	22 ± 6	15 ± 5	2.0 ± 0.4
b	C≡C—H	6.1 ± 0.7	1.0 ± 0.2	63 ± 5	11 ± 5			60 ± 21	11 ± 4
e	C=C—I	1.2 ± 0.1	34 ± 6						
f	I | C=C—H			3.5 ± 2.2	6 ± 5				
ATD	=O (no OH)							1800 ± 270	160 ± 32

[a] Compound **2a** is testosterone.

(Fig. 4), with a K_d for the low affinity ATD site of 2.9 ± 1.6 μM and a K_d for the high affinity site of 18 ± 4 nM. In contrast, binding to the individual liver and prostate cytosols could only be described by a single class of binding sites. These data are inconsistent with metabolism of ATD as an explanation for the different IC_{50} values observed in prostate and liver cytosol and suggest the existence of two distinct, tissue-specific, ATD binding sites.

In contrast to results obtained using ATD, L-598,229 produced IC_{50} values similar to the value measured for liver alone (data not shown). Furthermore, the data for the prostate/liver mixture were best fit to a theoretical curve that assumed only one class of binding sites, with a K_d for L-598,229 of 23 ± 3 nM. This value agrees well with the K_d value determined for both prostate and liver tissue cytosols alone (prostate K_d, 9 ± 4 nM; liver K_d, 49 ± 10 nM). Because the K_d values in the two tissues differ by only 4-fold, the inability to distinguish two distinct binding sites may reflect the small separation between these sites. Alternatively, these results may indicate that L-598,229 undergoes tissue-specific metabolism under the conditions of the assay, due to some component of the liver cytosol, or that liver cytosol

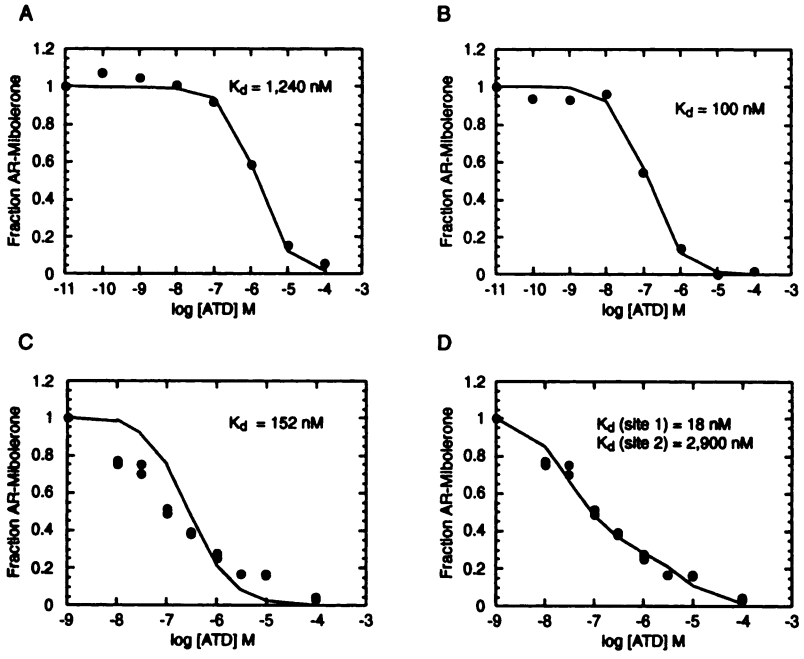


Fig. 4. A and B, Modeling of binding data obtained for ATD displacement of AR-[^{3}H]mibolerone complexes, using a one-site fit, for prostate (A) and liver (B) alone. C and D, Displacement of [^{3}H]mibolerone binding to prostate/liver cytosols at a ratio of 1:1, modeled using a one-site fit (C) or a two-site fit (D). The Marquardt algorithm was used and calculated K_d values are shown (24). The protein concentration for prostate alone was 0.59 mg/ml and that for liver alone was 2.0 mg/ml. Data shown are taken from a representative experiment.

contains a tissue-specific factor that interferes with the binding of L-598,229.

Determination of compound metabolism in tissue cytosols. The possibility of metabolism of the steroids used in this study was addressed further by resolving unlabeled competitor compounds, using reverse phase HPLC, before and after incubation in the presence of liver and prostate tissue cytosols. Fig. 5 shows a representative chromatogram for several of the competitor compounds used in the ligand specificity (competition curve) studies described above. In one experiment, incubation of ATD, mibolerone, testosterone, DHT, and TAC with either prostate or liver cytosols under conditions of the standard radioligand binding assay (overnight at 4°) produced either no detectable decrease or only a slight decrease in the amount of each compound extracted and isolated by reverse phase HPLC. In addition, no additional peaks in chromatograms from incubated cytosols could be detected, arguing against metabolism of these compounds during incubation in the presence of either cytosol. Results obtained after overnight incubation of L-598,229 and DHT in the presence of cytosolic extracts were difficult to interpret because of the small extinction coefficients of these steroids. Significantly, incubation of ATD in liver cytosol at 4°, 22°, and 32° appeared to produce no decrease in the amount of ATD extracted, within the apparent range of experimental error (Fig. 6). Thus, metabolism of ATD by a cytosolic enzyme is unlikely under the conditions of the binding assay carried out at 4°.

Cytochrome P-450 aromatase. Because ATD is a known inhibitor of cytochrome P-450 aromatase (7, 26), we explored the possible contribution of this enzyme to our binding data. Although aromatase is a membrane-bound enzyme, residual activity could be measured using cytosols. When necessary cofactors such as NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were added to the incubation mixture, approximately 23 fmol of the product [^{3}H]estrone/mg of soluble protein were estimated in dog liver after an overnight incubation at 4°. Much lower aromatase activity of ~0.6 fmol of product/mg of protein, or ~2.5% of "optimal" activity, was measured in TEGM buffer in the absence of added cofactors. The optimal activity of aromatase found in dog liver cytosol could be inhibited by ATD and by mibolerone, but only at relatively high concentrations, with IC_{50} values of ~1000 and 500 nM, respectively. In contrast, the IC_{50} for ATD inhibition of microsomal aromatase was found to be ~70 nM.[2] The affinities of mibolerone and ATD for the AR were thus >1700-fold and >6–10-fold greater, respectively, than those for the "cytosolic" aromatase when the aromatase assay was carried out in the presence of added cofactors and >67,000-fold and >200-fold greater, respectively, in the absence of added cofactors. It is concluded that aromatase contributes little or no binding to ATD or mibolerone under the conditions of the binding assay.

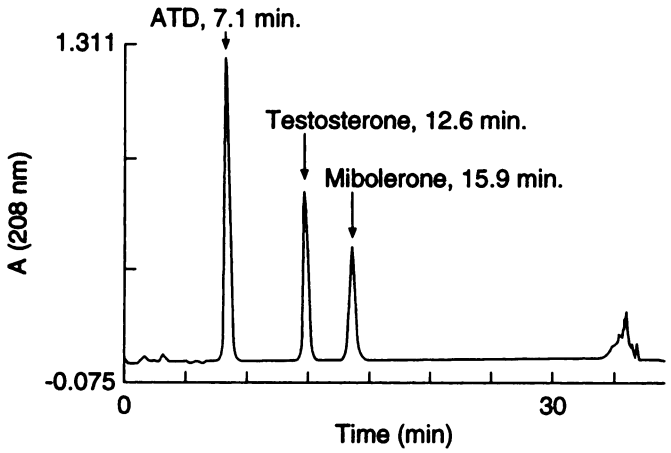


Fig. 5. HPLC chromatogram resolving the compounds ATD, testosterone, and mibolerone after incubation in the presence of prostate cytosol (overnight at 4°) and extraction, as described in Experimental Procedures. The peak eluting at ~36 min is observed with injections of mobile phase alone and is not due to the steroids injected. Data shown are taken from a representative experiment.

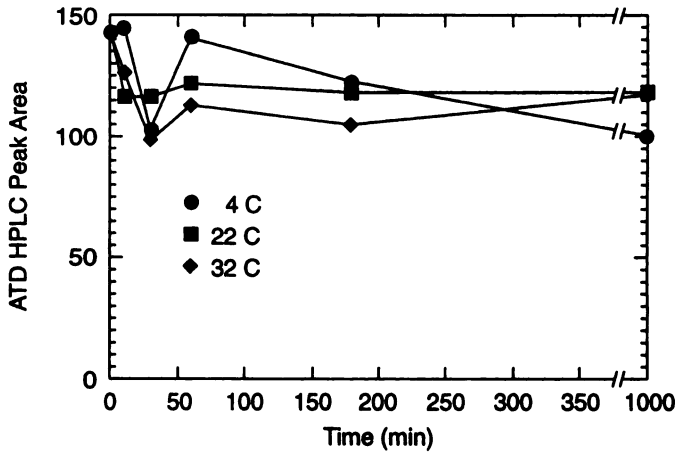


Fig. 6. Quantity of ATD extracted from liver cytosol after various times of incubation at 4°, 22°, or 32°, as detected at 208 nm. Data shown are taken from a representative experiment.

Discussion

A major unanswered question regarding the action of the androgenic signals DHT and testosterone is the mechanism by which these ligands regulate diverse biological functions via a single receptor. As a first step toward addressing this question, this study presents an extensive pharmacological survey of the AR/MBPs present in various dog cytosolic extracts, as well as the human AR-A and -B isoforms expressed in COS cells.

We attempted to distinguish the binding of DHT and testosterone to the AR/MBPs by using putative DHT- and testosterone-selective antagonists in competition with [^{3}H]mibolerone. Unlabeled mibolerone proved to be a useful benchmark in these studies, yielding an average IC_{50} of 0.32 ± 0.16 nM (12 experiments) for all five tissues in which binding could be detected. Estradiol was also found to have little or no tissue specificity in competing for [^{3}H]mibolerone binding, with an average IC_{50} of 7.7 ± 2.4 nM (six experiments). Estradiol has been shown to activate transcription of the Moloney murine leukemia virus-chloramphenicol acetyltransferase reporter gene via coexpressed AR even at 1 nM (28). Interestingly, the relative ability of DHT and of testosterone to compete for [^{3}H]mibolerone binding was found to differ from tissue to tissue. Binding in prostate exhibited a preference for binding of DHT, relative to testosterone, of ~8 ± 5-fold (four experiments, calculated as a ratio within indi-

[2] G. Harris and G. Cimis, personal communication.

vidual experiments), consistent with previously published studies using rat prostate (19, 21), human prostate (29), and human genital skin fibroblasts (30). Both isoforms of the human AR originally cloned from prostate were also found to exhibit an approximately 2–3-fold preference for binding of DHT, relative to testosterone. This binding ratio is also consistent with the ability of DHT and testosterone to activate transcription via the AR (28). In contrast, other tissues were found to have less preference for binding of DHT, relative to testosterone, ranging from a ~2–3-fold preference in kidney, liver, and testis to equipotent binding in adrenal gland.

The aforementioned observations of tissue-specific preferences for AR/MBP binding of DHT and testosterone were extended using the putative DHT antagonist L-598,229 and the testosterone antagonist ATD. L-598,229 was found to have high affinity for the AR/MBPs present in prostate, kidney, testis, and adrenal gland (2–7 nM) and much lower affinity for that in liver. ATD exhibited low affinity for the AR/MBPs present in prostate, adrenal gland, and testis, ranging from 2 to 3 μM. This value for ATD binding is consistent with that reported using rat brain cytosol (7). In contrast, ATD was found to have markedly higher affinity for binding to the AR/MBPs in both kidney and liver. Species that specifically bind [^{3}H]methyltrienolone (R118) or [^{3}H]mibolerone have been reported in rat liver and in cynomolgus monkeys, respectively (31–33), and have been proposed to play a role in hepatic protein secretion.

A search for structural analogues of ATD yielded a family of unsaturated androst-4-en-17β-ol-3-ones that also exhibit higher affinity binding to the AR/MBPs present in liver, relative to those present in prostate. Although these compounds are known to bind to ABPs (18), several lines of evidence argue against binding to ABPs rather than to the AR/MBPs in our studies. Firstly, mibolerone does not bind specifically to human ABP (21), so compounds that compete for high affinity [^{3}H]mibolerone binding are interacting with either the AR or a novel MBP, rather than with previously reported ABPs. In addition, the affinities of this class of compounds for the AR/MBPs in prostate or in liver exhibit a different structure-activity relationship than that observed for ABP (18). Compound **3a** (Δ^6-testosterone) binds to ABP about one third as strongly as does DHT. In contrast, **3a** is found to bind to the AR/MBPs present in dog liver approximately 200-fold more strongly than DHT. Unsaturation of the A- and B-rings of DHT markedly increases the affinity for the AR/MBPs, whereas these modifications decrease the affinity for binding to ABPs (18). Addition of an iodoethenyl group at position 17 of the steroid ring decreases binding to the AR/MBPs in liver, whereas this moiety has the opposite effect on binding to the ABP. Finally, the binding of mibolerone to purified dog SHBG was determined by displacement of [^{3}H]DHT using a filter binding assay (34, 35). Mibolerone was found to bind modestly to dog SHBG, with approximately 10-fold lower affinity than unlabeled DHT (data not shown). In contrast, the AR/MBPs present in dog prostate and liver bind to mibolerone with the same affinity or approximately 20-fold more strongly, respectively, relative to DHT.

Alternatively, the tissue-specific pharmacology of DHT, testosterone, L-598,229, and ATD could be a result of metabolism of these compounds under the conditions of the binding assay. To address this issue, the prostate and liver cytosols were mixed and binding to the mixture was analyzed. The results using ATD were consistent with the presence of two distinct and noninterconvertible mibolerone-binding populations present in prostate versus liver. Because the K_d values for ATD in the mixture did not approach the average of the two binding sites present in prostate and liver, it is not likely that tissue-specific cofactors interact with the AR/MBPs in these tissues. Whereas ATD is a known inhibitor of cytochrome P-450 aromatase, we found that this membrane-bound enzyme has no significant contribution to the binding of ATD in tissue cytosols. The binding data for prostate/liver mixtures were best fit to two distinct populations of binding sites, whereas binding to each individual tissue was described best by a single class of sites. In addition, no evidence for metabolism of compounds was observed by HPLC analysis after incubation in the presence of cytosolic extracts under the conditions of the binding assay. Thus, tissue-specific metabolism is not a likely explanation for the observed pharmacology of ATD. At this time, we cannot rule out metabolism of the series of unsaturated androst-4-en-17β-ol-3-ones tested as an explanation for higher affinity binding to the AR/MBPs in liver. Although it is unlikely, the relatively high affinity for ATD observed using liver cytosol could be due to a putative receptor distinct from the AR that binds specifically to ATD in rat liver cytosols (36). However, it is not known whether this putative receptor binds to [^{3}H]mibolerone with high affinity.

One possible explanation for the observation of two pharmacologically distinct AR/MBPs is differential splicing of the mRNA encoding the AR (8–10). The known examples of splicing variants of the AR either encode the same protein (8, 9) or do not affect the ligand binding domain (10, 11). To address this issue, polymerase chain reaction amplifications of the AR gene were carried out using a variety of both human and dog cDNAs and primers that span all eight known intron/exon boundaries. No evidence was found to support the existence of tissue-specific cDNAs (data not shown). Recently, a truncated form of the AR was reported (11). However, this second form of the human AR does not seem to account for the tissue-specific pharmacology observed using dog tissues, because human AR-A and -B exhibit similar binding profiles for all ligands tested. It is not known at present whether these two isoforms exist in dog tissues. Because it is unlikely that the AR is encoded by two genes or interacts with tissue-specific cofactors that could alter ligand binding, post-translational modification of the ARs expressed in different tissues appears to be a likely explanation for the differential pharmacological profiles observed in the present study. Alternatively, these data could support the existence of a novel mibolerone-binding species that is distinct in liver.

In summary, tissue-specific forms of the AR/MBPs have been characterized using a variety of putative DHT and testosterone antagonists and analogues as probes. Further characterization and purification of the AR/MBP species should aid in our understanding of the relative contribution of each protein to the specific signaling by DHT and testosterone. Such probes may be useful for separating the effects of DHT and testosterone on transcriptional activation of androgen-responsive genes, as well as on the actions of AR/MBPs *in vivo*.

Acknowledgments

The authors wish to thank Dr. Gary Rasmusson (Basic Chemistry, Merck Research Laboratories), Dr. Wesley Tanaka (Clinical Development, Merck Research Laboratories), Mr. Paul Cunningham (Lab Animal Resources, Merck Research Laboratories), and Dr. David Linemeyer and Dr. Michael R. Tota (Department of Molecular Pharmacology and Biochemistry, Merck Research Laboratories) for advice and support with this project. We also thank Prof. William Rosner (Roosevelt Hospital, New York, NY) for providing purified dog SHBG and Prof. Michael McPhaul (Southwestern Medical Center, Dallas, TX) for providing the human AR-B cDNA in the expression vector CMV-2.

References

1. Wilson, J. D., F. W. George, and J. E. Griffin. The hormonal control of sexual development. *Science (Washington D. C.)* **211**:1278–1284 (1981).
2. Tilley, W. D., M. Marcelli, and M. J. McPhaul. Recent studies of the androgen receptor: new insights into old questions. *Mol. Cell. Endocrinol.* **68**:C7–C10 (1990).
3. Sheridan, P. J. Can a single androgen receptor fill the bill? *Mol. Cell. Endocrinol.* **76**:C39–C45 (1991).
4. Katsumata, M., and A. S. Goldman. Separation of multiple dihydrotestosterone receptors in rat ventral prostate by a novel micromethod of electrofocusing. *Biochim. Biophys. Acta* **359**:112–129 (1974).
5. Goldsteyn, E. J., H. J. Goren, J.-G. Lehoux, and Y. A. Lefebvre. Phosphorylation and nuclear processing of the androgen receptor. *Biochem. Biophys. Res. Commun.* **171**:336–341 (1990).
6. Stamatiadis, D., F. Dadoun, M.-C. Portois, F. Wright, I. Mowszowica, and P. Mauvais-Jarvis. Isoelectric focusing and 2D electrophoresis of the human androgen receptor. *J. Steroid Biochem. Mol. Biol.* **41**:43–51 (1992).
7. Kaplan, M. E., and M. Y. McGinnis. Effects of ATD on male sexual behavior and androgen receptor binding: a reexamination of the aromatization hypothesis. *Horm. Behav.* **23**:10–26 (1989).
8. Faber, P., H. van Rooij, H. van der Korput, W. Baarends, A. Brinkmann, A. Grootegoed, and J. Trapman. Characterization of the human androgen receptor transcription unit. *J. Biol. Chem.* **266**:10743–10749 (1991).
9. McLachlan, R., B. Tempel, M. Miller, J. Bicknell, W. Bremmer, and D. Dorsa. Androgen receptor gene expression in the rat central nervous system: evidence for two mRNA transcripts. *Mol. Cell. Neurosci.* **2**:117–122 (1991).
10. Fischer, L., D. Cats, and D. Kelley. An androgen receptor mRNA isoform associated with hormone-induced cell proliferation. *Proc. Natl. Acad. Sci. USA* **90**:8254–8258 (1993).
11. Wilson, C. M., and M. J. McPhaul. A and B forms of the androgen receptor are present in human genital skin fibroblasts. *Proc. Natl. Acad. Sci. USA* **91**:1234–1238 (1994).
12. Wei, L. L., C. Gonzalez-Aller, W. M. Wood, L. A. Miller, and K. B. Horwitz. 5′-Heterogeneity in human progesterone receptor transcripts predicts a new amino-terminal truncated "C"-receptor and unique A-receptor messages. *Mol. Endocrinol.* **4**:1833–1840 (1990).
13. Geffroy-Roisne, S., J. Duval, and M.-L. Thieulant. Multiple forms of affinity-labeled estrogen receptors in rat distinct pituitary cells. *Endocrinology* **131**:1503–1510 (1992).
14. Baker, V. A., J. R. Puddefoot, S. Marsigliante, S. Barker, A. W. Goode, and G. P. Vinson. Oestrogen receptor isoforms, their distribution and relation to progesterone receptor levels in breast cancer samples. *Br. J. Cancer* **66**:1083–1087 (1992).
15. Martin, B., J.-M. Bernardon, M.-T. Cavey, B. Bernard, I. Carlavan, B. Charpentier, W. R. Pilgrim, B. Shroot, and U. Reichert. Selective synthetic ligands for human nuclear retinoic acid receptors. *Skin Pharmacol.* **5**:57–66 (1992).
16. Brendler, C. B., S. J. Berry, L. L. Ewing, A. R. McCullough, R. C. Cochran, J. D. Strandberg, B. R. Zirkin, D. S. Coffy, L. G. Wheaton, M. Hiler, G. D. Niswender, W. Scott, and P. C. Walsh. Spontaneous benign prostatic hyperplasia in the beagle: age associated changes in serum hormone levels, and the morphology and secretory function of the canine prostate. *J. Clin. Invest.* **64**:842–849 (1979).
17. Berry, S. J., J. D. Strandberg, D. S. Coffey. The development of canine benign prostatic hyperplasia in the beagle. *Prostate* **9**:363 (1986).
18. Diaz Cruz, P. J., N. S. Mason, B. J. Danzo, and H. E. Smith. Preparation of 17α-iodoethynyladrosta- and 17α-(2-iodoethynyl)adrosta-4,6-dien-17β-ol-3-ones as active site-directed ligands for androgen-binding proteins. *Steroids* **57**:569–576 (1992).
19. Schilling, K., and S. Liao. The use of radioactive 7α,17α-dimethyl-19-nortestosterone (mibolerone) in the assay of androgen receptors. *Prostate* **5**:581–588 (1984).
20. Bannister, P., P. Sheridan, and M. S. Losowsky. Use of a new radioactive ligand, 7α,17α-dimethyl-[17α-*methyl*-^{3}H]19-nortestosterone for the estimation of androgen receptors in rat liver cytosol. *J. Steroid Biochem.* **23**:121–123 (1985).
21. Traish, A. M., R. E. Müller, and H. H. Wotiz. Binding of 7α,17α-dimethyl-19-nortestosterone (mibolerone) to androgen and progesterone receptors in human and animal tissues. *Endocrinology* **118**:1327–1333 (1986).
22. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–249 (1976).
23. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
24. Duggleby, R. G. Regression analysis of nonlinear Arrhenius plots: an empirical model and a computer program. *Comput. Biol. Med.* **14**:447–455 (1984).
25. Orlowski, J., and A. F. Clark. An isocratic reversed-phase high performance liquid chromatographic analysis of 5α-reduced androgen metabolites formed by rat ventral prostate cells in culture. *J. Liquid Chromatogr.* **12**:1705–1718 (1989).
26. Cole, P. A., and C. H. Robinson. Mechanism and inhibition of cytochrome P-450 aromatase. *J. Med. Chem.* **33**:2933–2942 (1990).
27. Lubahn, D. B., D. R. Joseph, P. M. Sullivan, H. F. Willard, F. S. French, and E. M. Wilson. Cloning of human androgen receptor complementary DNA and localization to the X chromosome. *Science (Washington D. C.)* **240**:327–330 (1988).
28. Deslypere, J.-P., M. Young, J. D. Wilson, and M. J. McPhaul. Testosterone and 5α-dihydrotestosterone interact differently with the androgen receptor to enhance transcription of the MMTV-CAT reporter gene. *Mol. Cell. Endocrinol.* **88**:15–22 (1992).
29. Murthy, L. R., C. H. Chang, D. R. Rowley, P. T. Scardino, and D. J. Tindall. Physicochemical characterization of the androgen receptor from hyperplastic human prostate. *Prostate* **5**:567–579 (1984).
30. Grino, P. B., J. E. Griffin, and J. D. Wilson. Testosterone at high concentrations interacts with the human androgen receptor similarly to dihydrotestosterone. *Endocrinology* **126**:1165–1172 (1990).
31. Levinson, D. J., and D. E. Decker. Characterization of a [^{3}H]methyltrienolone (R1881) binding protein in rat liver cytosol. *J. Steroid Biochem.* **22**:211–219 (1985).
32. Sunahara, G. I., M. J. P. Finlayson, B. L. Warren, and G. D. Bellward. Characterization studies of a rat hepatic cytosolic androgen-binding protein. *Can. J. Physiol. Pharmacol.* **63**:952–957 (1985).
33. Marschke, K. B., and D. R. Koritnik. Estrogen and androgen receptors in the liver of cynomologus monkeys (*Macaca fascicularis*). *J. Steroid Biochem.* **26**:443–450 (1987).
34. Mickelson, K. E., and P. H. Petra. A filter assay for the sex steroid binding protein (SBP) of human serum. *FEBS Lett.* **44**:34–38 (1974).
35. Rosner, W. Some theoretical considerations regarding filter disk assay. *Anal. Biochem.* **67**:422–427 (1975).
36. Gustasfsson, J.-Å., Å. Pousette, Å. Stenberg, and Ö. Wrange. High-affinity binding of 4-androstene-3,17-dione in rat liver. *Biochemistry* **14**:3942–3948 (1975).

Send reprint requests to: J. Toney, Department of Biochemistry, Building 80N-A54, P.O. Box 2000, Merck Research Laboratories, Rahway, NJ 07065-0900.