7α-Methyl-17α-(E-2'-[^{125}I]iodovinyl)-19-nortestosterone: a new radioligand for the detection of androgen receptor

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We have synthesized two γ-emitting, ^{125}I-labeled steroids, E- and Z-7α-methyl-17α-(2'-[^{125}I]iodovinyl)-19-nortestosterone [^{125}I](E- and Z-MIVNT) for specific labeling of androgen receptors. [^{125}I]E- and [^{125}I]Z-MIVNT were synthesized stereospecifically from E- and Z-7α-methyl-17α-(2'-tri-n-butylstannyl-vinyl)-19-nortestosterone. The tin adducts were prepared by addition of tri-n-butyltin hydride to 7α-methyl-17α-ethynyl-19-nortestosterone, and after purification they were converted in high yield to the [^{125}I]MIVNT isomers by reaction with ^{125}I (generated in situ by oxidation of [^{125}I]iodide with chloramine T). The ^{125}I-labeled products were purified by high-performance liquid chromatography, and their mass determined with an ultraviolet detector (specific activity of both, approximately 2,200 Ci/mmol). In rat prostate cytosol, [^{125}I]E-MIVNT bound with high affinity to a single class of binding sites. Nonspecific binding in the presence of 5α-dihydrotestosterone was relatively low, and compared favorably with that obtained in parallel studies with [^{3}H]methyltrienolone (R1881). The E-isomer bound prostate cytosol with at least twice the affinity of the Z-isomer; therefore, the interaction of the E-isomer with the androgen receptor as well as other steroid receptors was studied in greater detail. Complexes of the androgen receptor with [^{125}I]E-MIVNT as well as [^{3}H]R1881 dissociate very slowly at 4C (k_{diss} for both = 0.04 h-1). Displacement studies showed that the interaction of [^{125}I]E-MIVNT with the androgen receptor is highly specific. Competition studies showed that unlabeled E-MIVNT binds poorly to other steroid receptors in rat tissue cytosols. These binding properties make [^{125}I]E-MIVNT a promising ligand for study of the androgen receptor, and [^{123}I]E-MIVNT a potential imaging agent for the detection of androgen-dependent tumors, such as prostate cancer. (Steroids **58**:13–23, 1993)

Keywords: androgen receptor ligand; steroid receptors; androgen analog; γ-emitting androgen; ^{125}I-labeled androgen; 7α-methyl-17α-(E-2'-[^{125}I]iodovinyl)-19-nortestosterone; steroids

Introduction

The search for selective agents to image steroid-sensitive tumors in vivo has led to the synthesis of several γ-emitting steroids.[1–12] In addition to their suitability for external imaging in vivo, these steroids, when labeled with ^{125}I, offer important advantages when compared with available ^{3}H-labeled steroid ligands, and have proven extremely useful for the quantitative measurement and visualization of specific steroid receptor populations in heterogeneous tissues. Because these ^{125}I-labeled ligands can be synthesized with specific activities of up to 2,200 Ci/mmol as compared with ~100 Ci/mmol for ^{3}H-labeled steroids, the sensitivity obtained in receptor assays with ^{125}I-labeled steroids is markedly greater than that obtained with ^{3}H-labeled ligands. Steroid ligands labeled with ^{125}I also have been used successfully to visualize steroid-concentrating cell groups and individual cells by autoradiographic techniques.[4,13–16] Auger electrons formed from ^{125}I decay have a higher energy than those released by ^{3}H decay, which, together with the higher specific activity possible with ^{125}I, allow autoradiographic visualization of steroid-concentrating cells using exposure times of <24 hours. In comparison, exposure times of several

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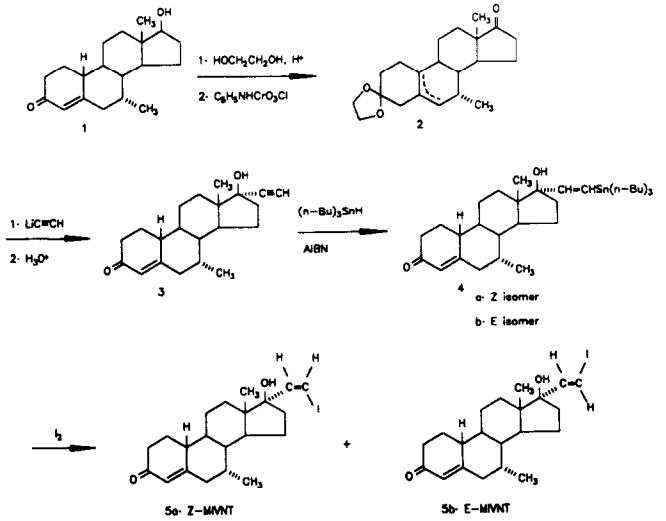

Figure 1 Synthetic scheme for E- and Z-MIVNT.

months to a year are typically required for ^{3}H-labeled ligands. Such shortened exposure times afforded by the ^{125}I label improve the practicality of quantitative steroid autoradiographic studies and can lead to improved combined autoradiographic/immunocytochemical studies as tissue antigens can be better preserved.[4]

Several ^{125}I-labeled steroid receptor ligands have been described. These include several estrogens,[1-10] progestins,[11,17,18] and a photoaffinity progestin receptor ligand.[12] Until now, a specific radioiodine-labeled androgen receptor ligand has not been demonstrated. The importance of obtaining such a probe for the visualization of prostate tumors has led to many attempts to synthesize γ-emitting androgens. The likelihood of obtaining an androgen receptor—mediated imaging agent has been demonstrated with ^{3}H androgens,[19] but this has been a difficult goal because the androgen receptor discriminates against analogs of androgens labeled with bulky isotopes. Consequently, previous attempts to design useful γ-emitting androgens have been unsuccessful.[20-25] We recently reported the synthesis of two iodinated analogs of 5α-dihydrotestosterone (5α-DHT), the E- and Z- isomers of 17α-(2-iodovinyl)-5α-DHT that bound the androgen receptor, albeit with much lower affinity than 5α-DHT.[26] Because the 17α-iodovinyl group did not completely block binding to the receptor, it appeared that this substituent, when combined with the proper steroid analog, might result in a useful radioiodine-labeled androgen receptor ligand. Because of the similarity to the potent androgen mibolerone (7α,17α-dimethyl-19-nortestosterone),[27] we chose to synthesize the E- and Z-isomers of the 17α-iodovinyl analog of 7α-methyl-19-nortestosterone (E- and Z-MIVNT). In this article we present the synthesis of these ^{125}I-labeled androgens (Figure 1) and further characterize the binding of the E-isomer to the androgen receptor and to other steroid hormone receptors.

Experimental

Steroids

[17α-Methyl-^{3}H]methyltrienolone (R1881; specific activity = 87.0 Ci/mmol), [2,4,6,7-^{3}H]estradiol (specific activity = 106.9 Ci/mmol), [17α-methyl-^{3}H]promegestone (R5020; specific activity = 84.7 Ci/mmol), [6,7-^{3}H]dexamethasone (DEX; specific activity = 44.7 Ci/mmol), unlabeled R1881, and unlabeled R5020 were obtained from Dupont Canada (Mississauga, Ontario, Canada). With the exception of MIVNT, all other steroids were purchased from Steraloids Inc. (Wilton, NH, USA). [^{3}H]R1881 was purified before use by partition chromatography on a Sephadex LH-20 column (Pharmacia, Piscataway, NJ, USA) using isooctane/methylene chloride/methanol (50:50:1) as the solvent system.[28]

Chemical synthesis of MIVNT

Melting points were obtained in a Koffler hot stage or in a Meltemp (Laboratory Devices, Cambridge, MA, USA) apparatus and are uncorrected. Infrared spectra were recorded in potassium bromide disks on a Beckman Acculab 4 spectrophotometer (Fullerton, CA, USA) or a Perkin Elmer model 1600 FT-IR instrument (Norwalk, CT, USA). Nuclear magnetic resonance (NMR) spectra were obtained at 100 MHz with a Bruker WP100SY FT instrument (Billerica, MA, USA). Mass spectra were recorded on Hewlett-Packard models 5985A and 5890A spectrometers (Palo Alto, CA, USA) at 70 eV with a direct-insertion probe. High-performance liquid chromatography (HPLC) was performed on a Beckman model 334 gradient system equipped with a model 421 controller, Altex CR-IA integrator-recorder, and Hitachi 100-10 variable wavelength detector (Danbury, CT, USA); ^{125}I-labeled steroids were chromatographed on a Waters modular system (Milford, MA, USA) consisting of a U6K injector, M-45 pump, and model 440 detector.

3,3-Ethylenedioxy-7α-methyl-5-estren-17-one and 3,3-ethylenedioxy-7α-methyl-5(10)-estren-17-one (2)

A mixture of 150 mg (0.521 mmol) of 7α-methyl-19-nortestosterone (**1**, Steraloids), 1.2 ml of ethylene glycol, 12 ml of benzene, and 14.0 mg of *p*-toluenesulfonic acid was refluxed for 43 hours. The mixture was cooled and transferred to a separatory funnel. The mixture was washed with 10 ml of saturated sodium bicarbonate, followed by three 10-ml portions of water, and dried over anhydrous sodium sulfate. Filtration and evaporation gave an oil that did not crystallize.

This oily product was oxidized by stirring with 0.81 g of pyridinium chlorochromate and 60 mg of anhydrous sodium acetate in 2 ml of methylene chloride for 5.5 hours at room temperature. The mixture was diluted with 50 ml of ether and the resulting orange-colored solution was filtered by gravity through a 1 cm deep bed of Florisil held in a 30-ml coarse frit funnel. The resulting colorless filtrate was evaporated to a clear oil (153 mg, 89%) which was a mixture of Δ^{5}- and $\Delta^{5(10)}$-isomers. Crystallization from acetone/petroleum ether, followed by recrystallization from absolute ethanol allowed isolation of 69.4 mg of the pure $\Delta^{5(10)}$-isomer: mp 137–140 C, Infrared (IR) (KBr) 1,733 cm^{-1}, NMR ($CDCl_3$) 3.95 (s, 4, ketal), 0.85 (s, 3, H-18) overlapping with 0.81 (d, 3, 7α-CH_3).

The residue remaining after evaporation of the mother liquor (83.6 mg) was a mixture of the Δ^{5}- and $\Delta^{5(10)}$-isomers and was free of any other contaminants as determined by thin layer chromatography (TLC) (3:1 benzene/ethyl acetate on silica gel).

7α-Methyl-17α-ethynyl-19-nortestosterone (3)

A solution of 153 mg of a mixture of the isomeric ketones (2) (0.46 mmol) in 7 ml of anhydrous dioxane was added to a suspension of 1.02 g (11.1 mmol) lithium acetylide/ethylenediamine complex in 7 ml of anhydrous dioxane in an atmosphere of acetylene gas. The mixture was stirred for 24 hours after which an additional 0.5 g of lithium acetylide was added and stirring continued for 60 hours. Saturated ammonium chloride (5 ml) was added to the mixture with cooling. The two-phase mixture was then diluted with 40 ml water and extracted with three 20-ml portions of ether. The combined ether extracts were washed with water, saturated sodium chloride, and dried over anhydrous sodium sulfate. Filtration and evaporation of solvent gave an oily residue of the ethynyl adduct. The C-3 ketal protecting group was hydrolyzed by stirring this residue in a mixture of 48 ml of dioxane and 12 ml of 2.0 N HCl at 37 C for 24 hours. The solution was neutralized by addition of 3.33 ml (24 mmol) of triethylamine, evaporated to 10 ml to remove dioxane, diluted with 75 ml distilled water, and extracted with three 25-ml portions of ether. The combined ether extracts were dried over anhydrous sodium sulfate, filtered, and evaporated to an oil that slowly crystallized. Recrystallization with acetone/petroleum ether gave 89.3 mg of pure product. An additional 13 mg of product was isolated by further workup of the mother liquors (overall yield 62%). mp 197–199 C; IR (KBr) 3,400 cm^{-1} 17β-OH, 3,248 ethynyl C-H, 2,114 C≡C, 1,655 C=O; NMR ($CDCl_3$) 5.8 (s, 1, H-4), 2.56 (s, 1, ethynyl H), 0.89 (s, 3, H-18), 0.76 (d, 3, J = 7 Hz, 7α-CH_3).

Z- and E-7α-methyl-17α-(2'-tri-n-butylstannylvinyl)-19-nortestosterone (4a and 4b)

A mixture of 31 mg (0.1 mmol) of 7α-methyl-17α-ethynyl-19-nortestosterone (3), 48 mg (0.165 mmol) of tri-n-butyltin hydride, 10.2 mg of azobisisobutyronitrile (AIBN), and 3.1 ml of dry benzene was placed in a screw-capped test tube, purged with dry nitrogen and heated at 80 C for 24 hours. After cooling, HPLC analysis of a 5-μl aliquot of this solution (25 cm × 4.6 mm LiChrosorb-Diol column, EM Science (Gibbstown, NJ, USA), 3 : 1 hexane/methylene chloride at 1 ml/min) showed a 2 : 1 ratio of the E- and Z-addition products migrating at 8.9 and 11.1 minutes, respectively. Approximately 300 μl of this solution was set aside for radioiodination (see below) and the remainder of the solution was iodinated directly as described below.

Z- and E-7α-methyl-17α-(2'-iodovinyl)-19-nortestosterone (Z- and E-MIVNT) (5a and 5b)

Iodine, 44 mg (0.17 mmol), dissolved in 0.9 ml of benzene was added to the solution of the mixture of Z- andE-7α-methyl-17α-(2'-tri-n-butylstannylvinyl)-19-nortestosterones (4a and 4b) described above. After stirring for 30 minutes, the mixture was diluted with 4.4 ml of a solution containing 10% sodium bisulfite and 1% potassium fluoride. The tube was capped and shaken. After removal of the aqueous layer, the organic solution was washed with two 4-ml portions of water, transferred to another tube, and dried over anhydrous sodium sulfate. Preparative HPLC was conducted on the mixture using a 25 cm × 1 cm silica column (Rainin, Woburn, MA, USA) (0.8% isopropanol in methylene chloride, 5 ml/min). This gave 4.5 mg (11.4% yield based on 3) of the Z-isomer, R_t = 16 minutes, and 10.6 mg (26.8% yield based on 3) of the E-isomer, R_t = 22 minutes (R_t = retention time).

Z-MIVNT. mp 60–65 C (acetone/petroleum ether); IR (KBr) 3,440 cm^{-1} (broad, 17β-OH), 1,660 (C=O); NMR ($CDCl_3$) 6.74 and 6.34 (AB pattern, 2, cis-CH=CH-, J = 8.7 Hz), 5.81 (s, 1, H-4), 0.90 (s, 3, H-18), 0.74 (d, 3, J = 7.1 Hz, 7α-CH_3); MS: 440 (1.2, parent), 313 (base, M-I), 295 (21, M-I-H_2O). High resolution mol wt for $C_{21}H_{29}O_2I$: calculated 440.1212, found 440.1225.

E-MIVNT. mp 76–79 C (acetone/petroleum ether); IR (KBr) 3,440 cm^{-1} (broad, 17β-OH), 1,660 (C=O); NMR ($CDCl_3$) 6.70 and 6.25 (AB pattern, 2, trans-CH=CH-, J = 14.5 Hz), 5.8 (s, 1, H-4), 0.93 (s, 3, H-18), 0.75 (d, 3, J = 7.1 Hz, 7α-CH_3); MS 440 (0.7, parent), 313 (base, M-I), 295 (23.4, M-I-H_2O). High resolution mol wt for $C_{21}H_{29}O_2I$: calculated 440.1212, found 440.1221.

7α-Methyl-17α-(E-2'-[^{125}I]iodovinyl)-19-nortestosterone

A 100-μl aliquot of the crude reaction mixture (the reaction mixture could be stored in the refrigerator for at least 6 months) containing ~300 μg of the Z- and E-17α-(2'-tri-n-butylstannyl) adducts (described above) was purified by HPLC on a 25 cm × 4.6 mm Lichrosorb-Diol column (EM Science) with 3 : 1 hexane-methylene chloride at 1 ml/min, and the fractions containing the Z- and E-isomers were collected (elution times as stated above).

The synthesis with radioiodide was performed in an enclosed hood outfitted with charcoal filters. The following synthetic procedure is representative of several similar reactions we have performed. The entirety of the fraction from the HPLC (above) containing the E-isomer of the tin adduct was evaporated and the residue in 100 μl of methylene chloride was added to a 300-μl Microflex reaction vial (Kontes Glass Co., Vineland, NJ, USA) into which a triangular micromagnetic stirring bar had been placed. This was layered with 25 μl of a 0.1 M phosphate buffer, pH 7.4, containing 40 μg (142 nmol) chloramine T. After the vial was closed with a cap containing a Teflon septum, a solution of 10 mCi (4.4 nmol) of [^{125}I]NaI (low pH, New England Nuclear Co., Boston, MA, USA) was injected through the septum into the mixture and the two phases were rapidly mixed on a magnetic stirrer for 30 min at room temperature. The reaction mixture was then quenched with 100 μl of an aqueous solution containing 10 mg of sodium bisulfite and 1 mg of KF.

After termination of the reaction, the vial was opened and the contents transferred to a 16 mm × 100 mm screw capped test tube with the aid of several washings with methylene chloride, a total volume of 4 ml. The aqueous layer was removed with a pipet and the organic phase was washed three times with 1-ml portions of water. The organic solution was dried over anhydrous sodium sulfate, filtered into a 10-ml pear-shaped flask through a Pasteur pipet plugged with glass wool and containing anhydrous sodium sulfate. The solution was evaporated to dryness on a rotary evaporator. The residue was dissolved in 200 μl of 2 : 3 methylene chloride/hexane and purified by HPLC on a 25 cm × 4.6 mm diol column (Waters) with 3 : 1 hexane-methylene chloride at a flow rate of 1 ml/min. The column was monitored with an ultraviolet (UV) detector at 254 nm. Fractions of 1 ml were collected and the content of ^{125}I determined in a γ counter. Most of the radioactivity eluted as a symmetrical peak; [^{125}I]E-MIVNT migrated at 27 minutes. In this system the ^{125}I-labeled steroid was cleanly separated from all other reactants. However, a UV absorbing peak that migrated just before or sometime in the beginning of the ^{125}I-labeled steroid peak was detected in every synthesis. Contamination with this UV-absorbing material did not affect binding of the ^{125}I-labeled steroid to the androgen receptor (as assessed by comparing different fractions from early and late in the ^{125}I-labeled steroid peak) and was therefore ignored. Those fractions containing radioactive E-MIVNT were combined, evaporated, and dissolved in 20 ml of 10% ethanol in benzene. The total radioactivity was determined to be 7.6 mCi,

a total radiochemical yield of 76%. The radioactive products were stored at 4 C in the alcoholic benzene solution. Analysis by thin-layer chromatography showed the materials to be stable under these conditions for a period of at least 2–3 months.

7α-Methyl-17α-(Z-2'-[^{125}I]iodovinyl)-19-nortestosterone

The ^{125}I-labeled Z-isomer was synthesized in the same manner as that used to produce the ^{125}I-labeled E-isomer. In the HPLC purification system described above, it eluted at 15 minutes.

Cytosol preparation

All cytosols were prepared from tissues removed from adult Sprague-Dawley CD-strain rats obtained from Charles River Breeding Laboratories (St. Constance, Quebec, Canada). For androgen-binding experiments, prostate glands obtained from male rats gonadectomized (GDX) 48 hours before being killed were homogenized in buffer TEGTMo [10 mM Tris, 1.5 mM Na_2EDTA, 10% (v/v) glycerol, 12 mM monothioglycerol, and 25 mM sodium molybdate, pH 7.4; 1.5 ml/prostate]. For glucocorticoid-binding experiments, liver extirpated from a female rat GDX and adrenalectomized (ADX) 1 week before being killed was homogenized in 5 vol buffer TEGTMo. For estrogen and progestin binding experiments, uteri from rats GDX 1 week before being killed were homogenized in buffer TEGT (TEGTMo without sodium molybdate; 2 ml/uterus). Uteri taken for progestin receptor binding were from animals treated with a 1.0-cm Silastic (Dow Corning, Midland, MI, USA) capsule containing crystalline estradiol diluted to 10% with cholesterol,[29] placed subcutaneously 3 days before being killed. Homogenates were centrifuged at 105,000 × *g* for 45 minutes at 0–4 C. The supernatants (cytosol) were stored at −85 C until assayed (<1 month). At the time of assay, frozen cytosol was allowed to thaw on ice in a 4 C room and was diluted with assay buffer before use. Protein concentration was adjusted to 6–10 mg/ml for prostate cytosol, and 0.8–1.2 mg/ml for uterine cytosol.

Receptor binding assays

Time course of association. Aliquots (600 μl) of prostate cytosol were incubated at 0–4 C with 100 μl buffer TEGTMo containing [^{125}I]E-MIVNT or [^{3}H]R1881 and 1 μM triamcinolone acetonide (TA) to suppress binding of the androgens to the progestin receptor,[30] in the presence or absence of a 500-fold molar excess of unlabeled 5α-DHT to assess nonspecific binding. Final incubate concentrations of the labeled androgens was 2.0 nM. At various times, bound steroid was separated from free steroid by gel filtration on 7 mm × 35 mm Sephadex LH-20 columns at 0–4 C.[31] At each time point, 100 μl of incubate was loaded onto the columns and washed into the column bed with 100 μl buffer TEGTMo. Thirty minutes after sample application, the macromolecular bound fraction was eluted with 400 μl buffer TEGTMo into 12 mm × 75 mm glass tubes ([^{125}I]E-MIVNT containing samples) or into scintillation counting vials ([^{3}H]R1881 containing samples). [^{125}I]E-MIVNT was counted at 70% efficiency using an ICN Micromedic 4/600 Plus γ counter (Huntsville, AL, USA). Tritium-labeled steroids were counted at 55% efficiency using an ICN Micromedic Taurus liquid scintillation counter after overnight extraction of radioligand into 5 ml Betacount (ICN) scintillation counting fluid.

Time course of dissociation. Aliquots (525 μl) of prostate cytosol were incubated at 0–4 C for 24 hours with 350 μl TEGTMo containing [^{125}I]E-MIVNT or [^{3}H]R1881 and 1 μM TA in the presence or absence of a 500-fold molar excess of unlabeled 5α-DHT. Final incubate concentration of the labeled androgens was 2.0 nM. After this incubation, 20 μl buffer TEGTMo containing unlabeled R1881 (1 μM final concentration) was added to all of the tubes and left at 0–4 C. To control for possible receptor degradation, a parallel set of controls that received 20 μl buffer without R1881 was included. At various times thereafter, macromolecular bound steroid was separated from free steroid by gel filtration on Sephadex LH-20 columns as described. Results are expressed as the percentage of specifically bound [^{125}I]E-MIVNT or [^{3}H]R1881, and were calculated as (specific binding in the presence of added 1.0 μM R1881/specific binding before addition of R1881) × 100.

Specificity of MIVNT binding. Binding affinities of E-MIVNT relative to reference compounds were determined for rat estrogen, androgen, glucocorticoid, and progestin receptors. For estrogen receptor binding, 75-μl aliquots of cytosol extracted from GDX rat uteri were incubated at 0–4 C for 20 hours with 50 μl TEGT buffer containing 2.0 nM [^{3}H]estradiol (final concentration) in the presence or absence of a range of different concentrations of unlabeled E-MIVNT, R1881, 5α-DHT, and estradiol-17β. For androgen receptor binding, aliquots of prostate cytosol obtained from GDX male rats were incubated at 0–4 C for 20 hours with buffer TEGTMo containing 2.0 nM [^{3}H]R1881 or [^{125}I]E-MIVNT in the presence or absence of a range of concentrations of unlabeled E-MIVNT, R1881, testosterone, 5α-DHT, estradiol, diethylstilbestrol (DES), progesterone, and corticosterone. For glucocorticoid receptor binding, aliquots of liver cytosol obtained from GDX/ADX rats were incubated at 0–4 C for 20 hours with buffer TEGTMo containing 3.0 nM [^{3}H]DEX in the presence or absence of a range of concentrations of unlabeled E-MIVNT, R1881, 5α-DHT, and DEX. For progestin receptor binding, aliquots of uterine cytosol obtained from GDX estrogen-treated rats were incubated at 0–4 C for 20 hours with TEGT containing 1.5 nM [^{3}H]R5020 in the presence or absence of a range of concentrations of unlabeled E-MIVNT, R1881, 5α-DHT, progesterone, and R5020.

Bound ^{3}H-labeled steroid was separated from free steroid by Sephadex LH-20 gel filtration, as previously described. Displacement curves were analyzed by a least-squares curve-fitting method with the use of the computer program ALLFIT.[32] Relative binding affinities (RBAs) were calculated based on the concentration of competitor required to displace saturable binding by 50%.

Saturation binding analysis. Aliquots (75 μl) of rat prostate cytosol were incubated at 0–4 C for 20 hours with 50 μl TEGTMo buffer containing a range of concentrations of [^{125}I]E-MIVNT or [^{3}H]R1881 and 1 μM TA, in the presence or absence of 1 μM 5α-DHT. Bound steroid was separated from free on Sephadex LH-20 columns as already described. A small aliquot (20 μl) of incubate was taken at the end of the incubation period to determine actual radioligand concentrations. The range of final radioligand concentrations was 0.02–5.5 nM for [^{125}I]E-MIVNT and 0.05–9.1 nM for [^{3}H]R1881. The resulting data were analyzed by the method of Scatchard using a computer-assisted nonlinear curve-fitting method adapted to an IBM-PC (Armonk, NY, USA) microcomputer.[33]

Results

Synthesis of [^{125}I]MIVNT

E- and Z-MIVNT(**5b** and **5a**) were synthesized as shown in Figure 1 in 21% overall yield. The starting material 7α-methyl-19-nortestosterone (**1**) was converted to its 3-ethylene ketal and oxidized to the 17-

ketone (**2**). Addition of lithium acetylide and hydrolysis of the ketal gave 7α-methyl-17α-ethynyl-19-nortestosterone (**3**). Addition of tri-n-butyltin hydride in the presence of initiator AIBN gave a 1 : 2 mixture of the 17α-(tri-n-butylstannylvinyl)-precursors **4a** and **4b**. This mixture was iodinated to give the products **5a** and **5b**, which were separated by preparative HPLC and characterized by standard analytical methods. In preparation of the ^{125}I-labeled steroid, the tin adducts **4a** and **4b** were separated by HPLC before radioiodination. Each isomer was treated with $^{125}I_2$ generated in situ from $^{125}I^-$ by the addition of chloramine-T. The no carrier–added ^{125}I-labeled products E- and Z-[^{125}I]MIVNT were purified by HPLC.

Time course of binding association and dissociation

Preliminary Scatchard and displacement binding studies in rat prostatic cytosols demonstrated E-MIVNT to have a twofold higher affinity for the androgen receptor than the Z-form (data not shown). Further studies of the properties of [^{125}I]E-MIVNT therefore focused on the E-isomer.

At 4 C, [^{125}I]E-MIVNT associated rapidly with androgen receptors in rat prostate cytosol, reaching a maximal level of saturable binding after 4 hours of incubation (Figure 2, top panel). In comparison, [^{3}H]R1881 associated at a slightly faster rate, reaching maximal levels after 2 hours of incubation (Figure 2, lower panel).

Dissociation of [^{125}I]E-MIVNT from androgen receptors was similar to that of [^{3}H]R1881, exhibiting apparent first-order kinetics for >12 hours at 4 C after addition of excess unlabeled competitor (data not shown). The dissociation rate constant (k_d) for [^{125}I]E-MIVNT was identical to that for [^{3}H]R1881 (0.04 h-1).

Stability of [^{125}I]E-MIVNT–bound complexes on Sephadex LH-20

In view of the relatively long interval between application of the incubate samples to the Sephadex LH-20 columns and final elution of the bound complexes, the possibility existed of artifactual losses of bound steroid with this method that might complicate interpretation of comparisons between the binding properties of different steroids. To determine the stability of [^{125}I]E-MIVNT–bound complexes in contact with Sephadex LH-20, prostate cytosol was incubated at 4 C for 20 hours with 2 nM [^{125}I]E-MIVNT and 1.0 μM TA in the presence or absence of 1.0 μM 5α-DHT. Samples were loaded onto Sephadex LH-20 columns as described in the Experimental section, and the macromolecular-bound fraction was eluted 15, 20, 25, 30, 40, 50, or 100 minutes after sample loading. For comparison, an identical parallel set of incubations was run using [^{3}H]R1881 as the ligand. The level of specifically bound [^{125}I]E-MIVNT was stable throughout the 100-minute test period, with the level of nonspecific [^{125}I]E-MIVNT binding decreasing as the length of exposure to LH-20 was increased, presumably as a result of selective

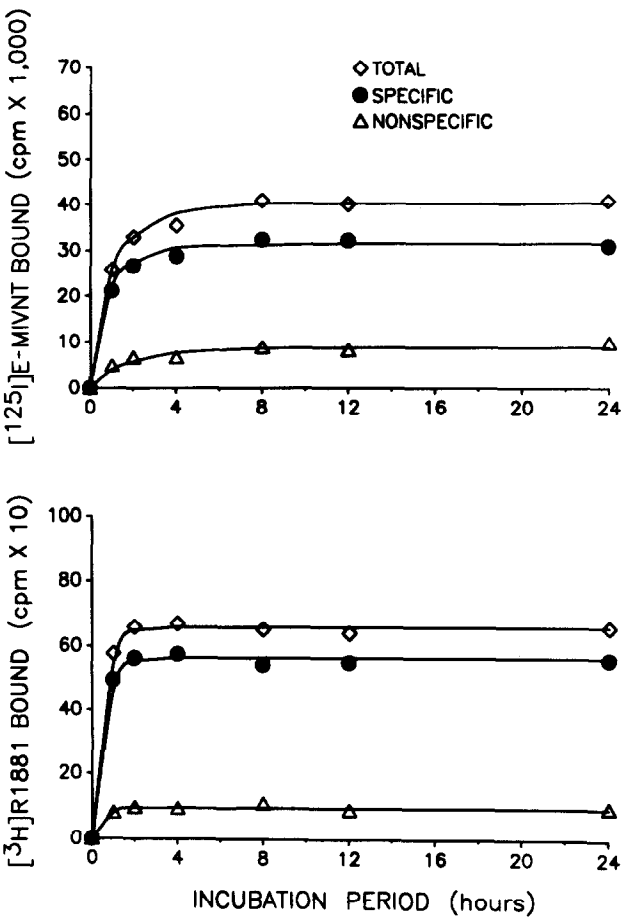

Figure 2 Comparison of the time course of association of [^{125}I]E-MIVNT and [^{3}H]R1881 binding to rat androgen receptor. Rat prostate cytosol was incubated at 0–4 C with 2.0 nM [^{125}I]E-MIVNT (top panel) or [^{3}H]R1881 (bottom panel) in the presence (nonspecific binding) or absence (total binding) of a 500-fold molar excess of 5α-DHT. TA (1.0 μM) was added to all incubations to suppress binding to the androgens to the progestin receptor. At various times, macromolecular bound steroid was separated from free steroid by gel filtration on Sephadex LH-20 columns. Specific binding was calculated as the difference between total and nonspecific binding. Values shown represent duplicate determinations.

dissociation of nonspecifically bound steroid in contact with the gel. In comparison, the concentration of specifically bound [^{3}H]R1881 was stable during the first 40 minutes of contact with Sephadex LH-20, but declined slightly (by about 25%) after 100 minutes of contact. In contrast to [^{125}I]E-MIVNT, the amount of nonspecific binding measured with [^{3}H]R1881 did not decrease with longer exposures to LH-20.

Specificity of [^{125}I]E-MIVNT binding

Competition assays were conducted to determine if E-MIVNT bound selectively to androgen receptors. Competition for the androgen receptor was conducted by incubating rat prostate cytosol for 20 hours at 4 C with 2 nM [^{125}I]E-MIVNT in the presence of a range of concentrations of 5α-DHT, E-MIVNT, estradiol,

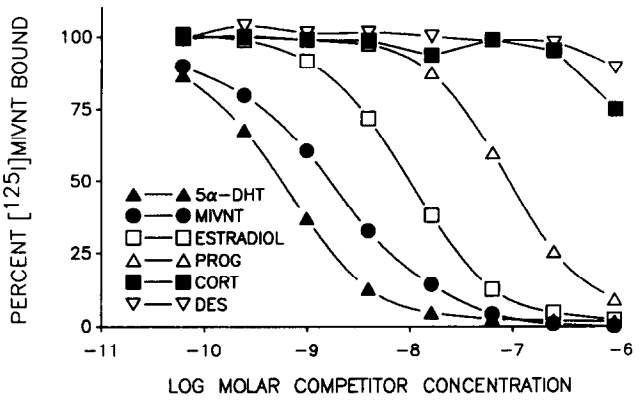

Figure 3 Competition of various ligands for [^{125}I]E-MIVNT binding sites in rat prostate cytosol. Cytosol was incubated at 0–4 C for 20 hours with 2.0 nM [^{125}I]E-MIVNT in the presence of various concentrations of radioinert 5α-DHT, E-MIVNT, estradiol, progesterone (PROG), corticosterone (CORT), or DES. Data are presented as the percentage of [^{125}I]E-MIVNT specifically bound in the absence of competitor. Nonspecific labeling was taken as the level of [^{125}I]E-MIVNT binding in the presence of 1.0 μM 5α-DHT. RBA values relative to 5α-DHT were 31.3 (E-MIVNT), 6.4 (estradiol), 0.697 (PROG), and <0.1 (CORT and DES).

progesterone, corticosterone, or DES. Of these compounds, 5α-DHT displaced [^{125}I]E-MIVNT binding most effectively, followed by E-MIVNT (Figure 3, top panel). Estradiol was relatively ineffective, whereas DES did not compete for [^{125}I]E-MIVNT binding at all. Progesterone and corticosterone were found to compete very weakly for [^{125}I]E-MIVNT binding sites.

To compare the binding of E-MIVNT with R1881 and the major endogenous ligands for the androgen receptor, 5α-DHT and testosterone, parallel competition assays were run using either [^{3}H]R1881 or [^{125}I]E-MIVNT as the labeled ligand. All incubations contained 1.0 μM TA in order to prevent binding of the androgens to the progestin receptor.[30] A similar pattern of displacement was obtained with both labeled compounds (Table 1). Of the four competitors, R1881 was the most effective, followed in turn by 5α-DHT, testos-

Table 1 RBAs obtained when [^{3}H]R1881 or [^{125}I]MIVNT are used as the radiolabeled ligand

Competitor	Radiolabeled ligand: [^{3}H]R1881 RBA	Radiolabeled ligand: [^{125}I]E-MIVNT RBA
R1881	100	100
5α-DHT	53 ± 3	48 ± 6
Testosterone	60 ± 4	46 ± 7
E-MIVNT	12 ± 1	11 ± 1

Rat prostate cytosol was incubated at 4 C for 20 hours with 2.0 nM [^{3}H]R1881 or [^{125}I]MIVNT in the presence of 1 μM triamcinolone acetonide and varying concentrations of radioinert R1881, MIVNT, 5α-DHT, or testosterone. The resulting competition curves were determined and analyzed by Allfit[32] to obtain RBA values ± SE.

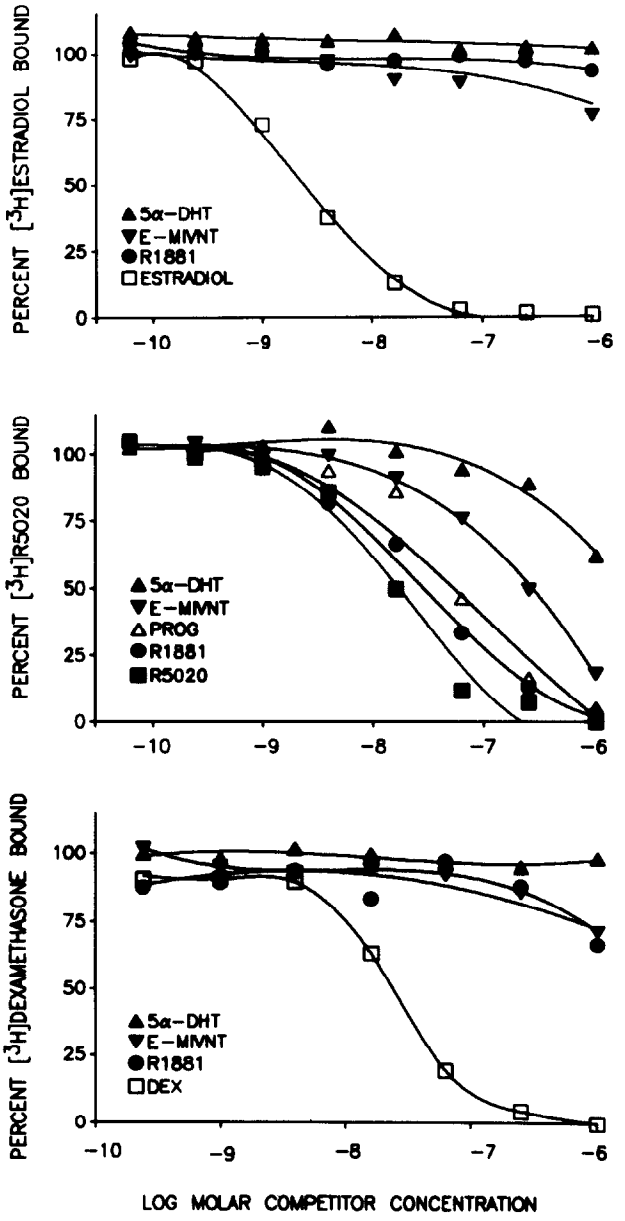

Figure 4 Top panel: Competition of E-MIVNT, R1881, and 5α-DHT for binding to [^{3}H]estradiol binding sites in rat uterine cytosol. Cytosol was incubated at 0–4 C for 20 hours with 2.0 nM [^{3}H]estradiol in the presence of various concentrations of radioinert estradiol, 5α-DHT, E-MIVNT, or R1881. Data are presented as the percentage of [^{3}H]estradiol specifically bound in the absence of competitor. Nonspecific labeling was taken as the level of [^{3}H]estradiol binding in the presence of 1.0 μM estradiol. Middle panel: Competition of E-MIVNT, R1881, and 5α-DHT for binding to [^{3}H]R5020 binding sites in rat uterine cytosol. Cytosol from GDX rats was incubated at 0–4 C for 20 hours with 2.0 nM [^{3}H]R5020 in the presence of various concentrations of radioinert progesterone, R5020, 5α-DHT, E-MIVNT, or R1881. Data are presented as the percentage of [^{3}H]R5020 specifically bound in the absence of competitor. Nonspecific labeling was taken as the level of [^{3}H]R5020 binding in the presence of 1.0 μM R5020. Bottom panel: Competition of E-MIVNT, R1881, and 5α-DHT for binding to [^{3}H]DEX binding sites in rat liver cytosol. Cytosol from ADX rats was incubated at 0–4 C for 20 hours with 2.0 nM [^{3}H]DEX in the presence of various concentrations of radioinert DEX, 5α-DHT, E-MIVNT, or R1881. Data are presented as the percent of [^{3}H]DEX specifically bound in the absence of competitor. Nonspecific labeling was taken as the level of [^{3}H]DEX binding in the presence of 1.0 μM DEX.

Table 2 Calculated RBAs of MIVNT for steroid hormone receptors as determined by competition binding assays

Steroid receptor type	RBA (%)		
	E-MIVNT	R1881	5α-DHT
Androgen receptor[a]	23.3–34.3	210.8	100
Estrogen receptor[b]	<0.1	<0.1	<0.1
Glucocorticoid receptor[c]	<0.1	<0.1	<0.1
Progestin receptor[d]	7.0	53.8	0.9

RBAs are based on the concentration of competitor required to displace saturable binding by 50% and were calculated as

$$\frac{\text{concentration of reference competitor resulting in 50\% displacement}}{\text{concentration of test competitor resulting in 50\% displacement}} (\times 100).$$

[a] Rat prostate cytosol incubated for 20 hours at 4 C with 2.0 nM [^{125}I]E-MIVNT with or without unlabeled competitor (RBA relative to 5α-DHT).
[b] Rat uterine cytosol incubated for 20 hours at 4 C with 2.0 nM [^{3}H]estradiol with or without unlabeled competitor (RBA relative to estradiol).
[c] Rat liver cytosol incubated for 20 hours at 4 C with 3.0 nM [^{3}H]DEX with or without unlabeled competitor (RBA relative to DEX).
[d] Rat uterine cytosol incubated for 20 hours at 4 C with 1.5 nM [^{3}H]R5020 with or without labeled competitor (RBA relative to R5020).

terone, and E-MIVNT. Identical results were obtained when the incubation time was shortened from 20 to 4 hours, suggesting that the displacement curves did indeed reflect the condition at equilibrium (data not shown).

Competition for the estrogen receptor was conducted by incubating rat uterine cytosol for 20 hours at 4 C with 2 nM [^{3}H]estradiol in the presence of various concentrations of R1881, E-MIVNT, 5α-DHT, and estradiol. Of these compounds, only estradiol was found to displace [^{3}H]estradiol binding from rat uterine cytosol (Figure 4, top panel).

Competition for the progestin receptor was conducted by incubating uterine cytosol from estrogen-treated GDX rats at 4 C for 20 hours with 1.5 nM [^{3}H]R5020 in the presence of various concentrations of R1881, E-MIVNT, 5α-DHT, progesterone, and unlabeled R5020. Of these compounds, R5020 displaced [^{3}H]R5020 binding most effectively (Figure 4, middle panel). R1881 was also an effective competitor, with a relative binding affinity close to that of progesterone. In comparison, E-MIVNT exhibited only weak competition for [^{3}H]R5020 binding. Consistent with previous studies, 5α-DHT did not exhibit measurable affinity for the progestin receptor.[34]

Competition assays for the glucocorticoid receptor were conducted by incubating liver cytosol from ADX female rats with 3.0 nM [^{3}H]DEX in the presence of a range of concentrations of R1881, E-MIVNT, 5α-DHT, and unlabeled DEX. None of the three androgens displaced [^{3}H]DEX binding appreciably (Figure 4, bottom panel).

The relative binding affinities for the interactions of E-MIVNT, R1881, and 5α-DHT with different classes of steroid receptor are summarized in Table 2.

Affinity of [^{125}I]E-MIVNT for androgen receptors

In view of the relatively small differences noted between [^{125}I]E-MIVNT and [^{3}H]R1881 with respect to their rates of association and dissociation for binding to prostatic androgen receptors (Figure 2), our finding of an approximately sixfold difference in relative binding affinity between the unlabeled forms of these two compounds (Table 1) was unexpected. Saturation binding assays were therefore conducted to directly determine the equilibrium dissociation constants of [^{125}I]E-MIVNT and [^{3}H]R1881 for rat prostate androgen receptors. Prostate cytosol was incubated at 4 C for 20 hours with a range of concentrations of [^{125}I]E-MIVNT or [^{3}H]R1881 in the presence of 1.0 μM TA. Nonspecific binding was measured at each concentration in the presence of 1.0 μM DHT. Saturation of rat prostate cytosol was reached with a concentration of 1.0–2.0 nM [^{125}I]E-MIVNT, approximately the same concentration as that required for [^{3}H]R1881 (Figure 5). Due to the much higher specific activity of [^{125}I]E-MIVNT and the greater counting efficiency possible with ^{125}I, count rates at saturation were much greater with [^{125}I]E-MIVNT than with [^{3}H]R1881 (120,000 versus 4,500 cpm). Scatchard representation of the data (Figure 5) showed [^{125}I]E-MIVNT binding to a single saturable binding site with a k_d of 0.079 nM, with only a slightly lower k_d obtained for [^{3}H]R1881 (0.037 nM).

The slightly greater than twofold difference in affinity between E-MIVNT and R1881 observed in the Scatchard analyses was consistent with the association and dissociation rate data, but contrasted with the results from the displacement curves (Table 1). We considered two possible interpretations of these findings: either the properties of the [^{125}I]E-MIVNT preparation were different from those of the corresponding unlabeled steroid, or there might be an experimental artifact in the displacement studies. The latter possibility was suggested by our experience in handling aqueous solutions of [^{125}I]E-MIVNT, which showed a tendency for this steroid to adsorb to glass and plastic surfaces. For the Scatchard isotherms (Figure 5) the actual steroid concentrations remaining in the incubation mixtures at the time of separation of the bound steroid could be determined precisely. For the displacement curves, however, the final concentrations of the unlabeled competitors present in solution could not actually be measured. It therefore seemed possible that the displacement experiments might have underestimated the real receptor affinity of E-MIVNT. To explore this possibility further, the ability of radioinert R1881 and E-MIVNT to displace [^{3}H]R1881 binding to rat prostate cytosol was reexamined using a different experimental design, in which we could directly estimate the extent to which the unlabeled competitor might be lost. Rat

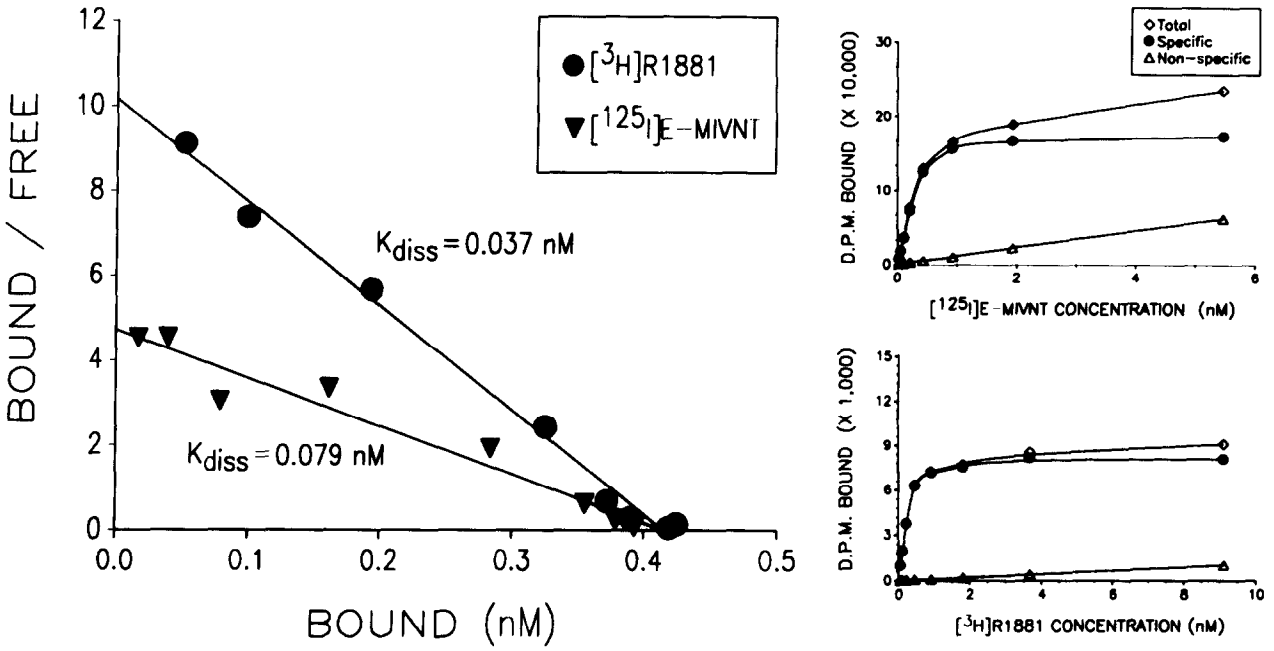

Figure 5 Saturation binding analysis of [^{125}I]E-MIVNT and [^{3}H]R1881 binding to rat prostate cytosol. Cytosol was incubated at 4 C for 20 hours with a range of concentrations of [^{125}I]E-MIVNT or [^{3}H]R1881 in the presence of 1.0 μM TA. Nonspecific binding was measured at each concentration in the presence of 1.0 μM DHT. Saturation binding curves for each of the radiolabeled androgens are shown on the right. Scatchard representation of the data is shown on the left.

prostate cytosol was incubated for 20 hours at 4 C with increasing concentrations of [^{3}H]R1881 in the presence of 0.5 nM R1881 or 2.0 nM E-MIVNT. All tubes contained 1.0 μM TA. Parallel incubations were also performed containing the same volumes of cytosol and buffer, in which the range of [^{3}H]R1881 concentrations was omitted, but the unlabeled competitors were mixed with a small quantity of the homologous labeled steroid, to allow determination of procedural losses. Scatchard analysis of the binding data thus obtained are shown in Figure 6 (top panel). As expected, both unlabeled steroids competed with [^{3}H]R1881, decreasing its apparent affinity for the androgen receptor without significantly affecting the binding capacity. The parallel, control incubations showed no evidence of procedural losses for R1881. In contrast, a substantial loss of E-MIVNT was observed during the preparation of the incubation mixtures, the final concentration of this steroid at the end of the incubations representing only 1.0 nM, 50% of the target 2.0-nM concentration. Calculation of apparent relative binding affinities based on the decrease in slope of the Scatchard plots and the competitor concentration estimated to be present in the incubations gave a value of 34% for E-MIVNT (R1881 = 100%).

The apparent loss of E-MIVNT noted in this experiment indicated that the previous displacement data for the progestin receptor (Figure 4 and Table 1) might similarly have underestimated the apparent binding affinity of this compound compared with R1881. Therefore, Scatchard plots in the presence and absence of unlabeled R1881 and E-MIVNT were repeated with [^{3}H]R5020 as the label in rat uterine cytosol (Figure 6, lower panel). Once again, the final concentration of E-MIVNT was found to represent only ~50% of the target value, in contrast to R1881, for which procedural losses amounted to <10%. The calculated relative binding affinity for E-MIVNT for this experiment based on the concentrations of competitor estimated to be present in solution at equilibrium was 24% of that for R1881, approximately twice the value calculated previously from the displacement curves (13%; Figure 4 and Table 1).

Discussion

The development of specific, high-affinity, radioiodine-labeled ligands for the androgen receptor may make possible the imaging and detection of androgen-dependent tumors, such as prostate cancer, as well as greatly facilitate the study of androgen action in heterogeneous tissues. The increase in sensitivity of conventional biochemical assays afforded by ^{125}I-labeling should facilitate receptor measurement in small tissue samples, such as needle biopsy samples. These ligands may also prove useful for quantitative autoradiographic analysis of androgen receptor distributions in tissue sections.[35] Several studies have demonstrated heterogeneity in the distribution of androgen receptors in the prostate gland, interactions between androgen-sensitive and androgen-insensitive cell types being important for androgen-induced prostatic growth and differentiation.[35,36] Similarly, in the brain, androgen-responsive cells appear not to be evenly distributed, but are localized

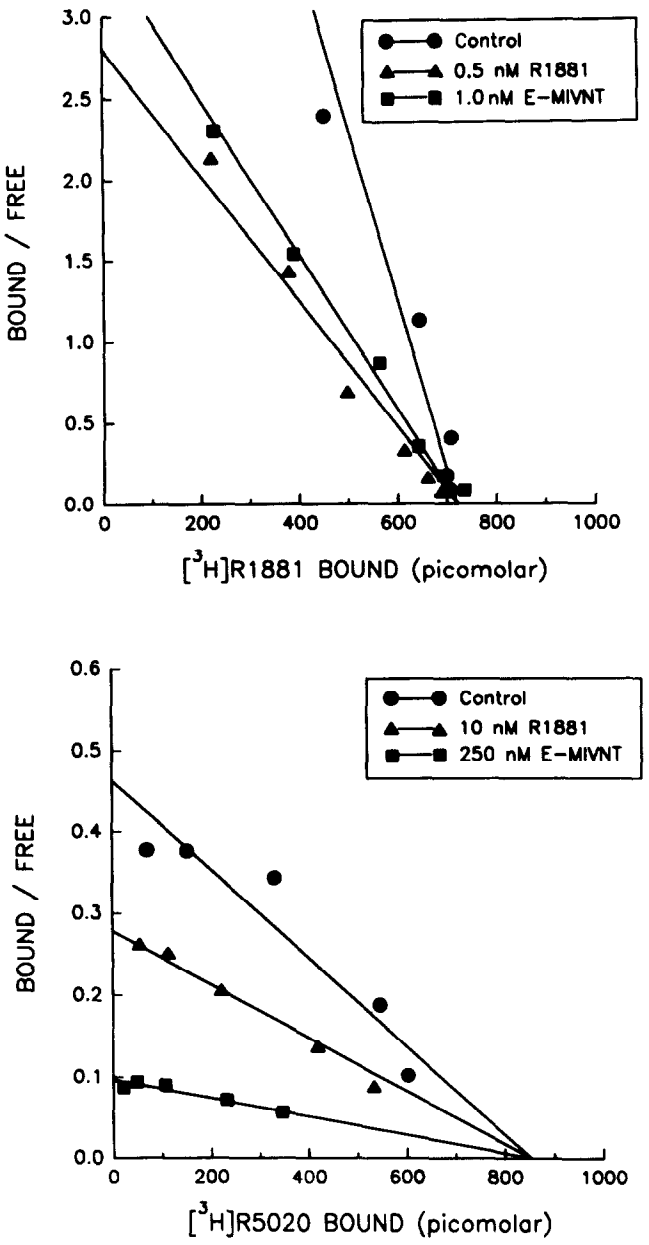

Figure 6 Top panel: Ability of radioinert R1881 and E-MIVNT to displace [^{3}H]R1881 binding to rat prostate cytosol. Cytosol was incubated at 4 C for 20 hours with increasing concentrations of [^{3}H]R1881 in the presence of 0.5 nM R1881 or 2.0 nM E-MIVNT. Nonspecific binding was determined in incubations containing 1.0 μM 5α-DHT. TA (1.0 μM) was added to all incubations to suppress binding to the progestin receptor. Parallel incubations in which [^{3}H]R1881 was omitted and to which a trace of the homologous radiolabeled ligand was added showed that 50% of the E-MIVNT was lost by adsorbing to glass and plastic surfaces. Thus, the final concentration of unlabeled E-MIVNT was 1.0 nM. Based on the shift in the apparent binding affinity resulting from the inclusion of the radioinert ligands, the apparent relative binding affinity for E-MIVNT was 34% (R1881 equals 100%). Bottom panel: Ability of radioinert R1881 and E-MIVNT to displace [^{3}H]R5020 binding to rat uterine cytosol. Cytosol was incubated at 4 C for 20 hours with increasing concentrations of [^{3}H]R5020 in the presence of 10.0 nM R1881 or 500 nM E-MIVNT. Nonspecific binding was determined in incubations containing 1.0 μM R5020. Parallel incubations in which [^{3}H]R5020 was omitted and to which a trace of the homologous radiolabeled ligand was added showed that 50% of the E-MIVNT was lost by adsorbing to glass and plastic surfaces. Thus, the final concentration of unlabeled E-MIVNT was 250.0 nM. Based on the shift in the apparent binding affinity resulting from the inclusion of the radioinert ligands, the relative binding affinity of E-MIVNT for the progesterone receptor was 24% of that of R1881.

in specific areas, primarily the hypothalamic/preoptic area and limbic system.[37] Even within these regions, androgen-sensitive neurons are juxtaposed with a large number of non–androgen-sensitive cells. To begin to understand how androgen action in the brain results in changes in neural function, attention must be focused on these specific groups of cells, or ideally, on individual cells. Autoradiographic visualization of steroid uptake offers the necessary anatomical resolution, but has hitherto been hampered by the long exposure times necessary with commercially available ^{3}H-labeled ligands. The use of ^{125}I-labeled ligands shortens the exposure times to the point that these techniques can be used on a routine basis.[13,14]

The experiments presented in this study demonstrate that E-MIVNT possesses key binding characteristics that enable its use for the quantification of androgen binding sites. This compound can be readily synthesized with a specific activity of up to 2,200 Ci/mmol and exhibits both high affinity and selectivity for the androgen receptor. [^{125}I]E-MIVNT has androgen-binding properties similar to those of [^{3}H]R1881, binding rapidly to the unoccupied androgen receptor and dissociating slowly at 0–4 C. Competition binding studies indicated that E-MIVNT does not compete appreciably for binding to either the glucocorticoid or estrogen receptor (Table 2). E-MIVNT does bind somewhat to the progestin receptor; however, this binding is relatively weak. In comparison, R1881 exhibits a fourfold higher affinity than E-MIVNT for the rat uterine progestin receptor, binding just about as well as the endogenous ligand, progesterone. This is consistent with other studies examining the affinity of R1881 for the progesterone receptor.[19,30,34] The lower affinity of E-MIVNT for the progesterone receptor may be due in part to the presence of a 7α-methyl group, which decreases the affinity of 19-nor steroids for this receptor.[38]

While this article was in preparation, a study by Salman and Chamness reported the synthesis and binding affinity of radioinert E-MIVNT for the androgen receptor.[39] These investigators reported a very similar relative binding affinity for the androgen receptor (34% relative to DHT, as compared with our values of 23–34%, compared with DHT). Although the synthetic method used by these investigators is essentially the same as reported here, they apparently did not detect the presence of the Z-tin adduct (**4a**) or its iodinated product, Z-MIVNT (**5a**).

In contrast to the relative binding affinity data obtained using competition curves, Scatchard analyses using [^{125}I]E-MIVNT indicated a much smaller differ-

ence in affinity between E-MIVNT and R1881. Whereas the competition data indicated RBAs for E-MIVNT of ~30% relative to 5α-DHT, as compared with 210% for R1881, only a twofold difference in affinity was observed between [^{125}I]E-MIVNT and [^{3}H]R1881 using Scatchard plots. The reason for this disparity appears to be that, with the labeled ligands, an accurate measure of the concentration of steroid in solution can be obtained, whereas with the unlabeled compounds the assumption is made that the free steroid concentration at equilibrium is the same as that originally added to the tubes. This assumption is valid for most hormonal steroids in incubations with tissue extracts: we have previously used a wide variety of steroids (including estradiol, R5020, progesterone, DEX, and 5α-DHT) for binding studies under identical conditions to those used here, without encountering significant adsorption problems. With [^{125}I]E-MIVNT, however, we found that substantial quantities of the tracer adsorbed to the surfaces of the glass and plastic materials used in preparing the incubation mixtures. This did not affect interpretation of the Scatchard plots, because samples were taken at the end of the incubations to determine the concentrations of radiolabeled ligand still present in solution. However, for the displacement curves the actual concentrations of ligand present at equilibrium could not be determined. From the data obtained with Scatchard plots in the presence and absence of unlabeled R1881 and E-MIVNT, it is likely that displacement curves based on the added concentrations of unlabeled competitor underestimate the relative affinity of E-MIVNT by at least twofold.

Because of its high affinity and selectivity for the androgen receptor, E-MIVNT may prove useful for the in vivo imaging of androgen-sensitive tissues, including prostate tumors. Although other androgen analogs have been evaluated as prostate-imaging agents, their affinity for the androgen receptor has not been sufficient to allow in vivo imaging of the androgen receptor.[21–25] Another difficulty encountered with in vivo imaging of androgen receptors is obtaining adequate exchange of receptor-bound endogenous ligands with the radioligand. This may not be a problem for MIVNT, because the affinity of this compound for human androgen receptors may be even higher than that for the rat. Salman and Chamness[39] have reported that MIVNT possesses an affinity for the human prostate androgen receptor that exceeds that of 5α-DHT (RBA 120%). Studies are now ongoing to determine if this apparent higher affinity of MIVNT may facilitate exchange labeling of human androgen receptors and thus enable in vivo imaging of androgen-concentrating cells in humans.

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