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Affinity Labeling of the Androgen Receptor with Nonsteroidal Chemoaffinity Ligands

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ABSTRACT. We synthesized a series of potential chemoaffinity ligands for the androgen receptor (AR) by means of structural modifications of bicalutamide, a known nonsteroidal antiandrogen used in the treatment of hormone-dependent prostate cancer. We determined AR binding affinities of these ligands, identified chemoaffinity ligands by exchange assays, and confirmed irreversible binding to the AR by Scatchard analyses. AR binding affinity was determined in a competitive binding assay with a radiolabeled high-affinity AR ligand, [^{3}H]mibolerone ([^{3}H]MIB). For exchange assays, AR were incubated with an excess of each ligand, and then adsorbed onto hydroxyapatite (HAP). HAP-bound AR then were incubated with [^{3}H]MIB to determine the remaining exchangeable specific binding sites. To determine the concentration of binding sites (B_{max}), using Scatchard analysis, AR were incubated with a fixed concentration of ligand and increasing [^{3}H]MIB concentrations. The ligands showed a wide range of AR binding affinities. In the exchange assays, three isothiocyanate derivatives of *R*-bicalutamide, the *p*-isothiocyanate (*R*-4), the *p*-thio-isothiocyanate (*R*-6), and the *m*-isothiocyanate (*R*-3), reduced exchangeable specific binding of [^{3}H]MIB by 85, 84, and 50%, respectively. The *S*-isomer of *p*-thio-isothiocyanate (*S*-6), which showed 700-fold lower AR binding affinity than *R*-6, did not reduce exchangeable specific binding of [^{3}H]MIB. In Scatchard analyses, the isothiocyanate derivatives *R*-3, *R*-4, and *R*-6 showed significant and progressive reduction in B_{max} at increasing concentrations. The results indicate that initial specific reversible AR binding was required for subsequent covalent labeling, and that *R*-3, *R*-4, and *R*-6 bound the AR specifically and irreversibly. These isothiocyanate derivatives of *R*-bicalutamide are the first specific chemoaffinity ligands for the AR, and will provide valuable tools for the molecular characterization of the ligand binding domain of the AR. BIOCHEM PHARMACOL **58**;8:1259–1267, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. affinity labels; androgen receptor; ligand binding domain; antiandrogens; nonsteroidal ligands; Scatchard analysis

Affinity labeling is the process by which ligand-binding receptors are labeled covalently by reactive analogs of chemically modified reversible ligands. This process generally is considered to be a labeling method with high selectivity [1], since the affinity labels often retain the binding selectivity of the reversible ligands. It is an extremely useful technique for studying the ligand binding domain(s) of a receptor, and especially for mapping the amino acid contact points of ligands in the regions of the receptor involved in ligand binding. Such information is valuable for receptors whose primary structure is known but whose secondary and tertiary structures are still undetermined.

Useful affinity ligands for steroid hormone receptors have been of two types, photoreactive ligands and chemoaffinity ligands [2]. Chemoaffinity ligands such as tamoxifen aziridine and dexamethasone 21-mesylate have been used successfully to covalently label the estrogen receptor [3] and the glucocorticoid receptor [4], respectively. Affinity labeling of the AR§ has not been successful due to the unavailability of a specific chemoaffinity ligand, and to the extremely low efficiency of covalent bond formation (0.4 to 6.8%) with the synthetic photoreactive ligand [^{3}H]methyltrienolone [5–8]. Previously reported steroidal analogs that were designed as chemoaffinity ligands for the AR were not successful [9, 10].

In this report, we synthesized electrophilic nonsteroidal bicalutamide analogs as potential AR chemoaffinity ligands, including isothiocyanate (*R*-3 to *R*-6 in Table 1) and α-haloacetamide derivatives (*R*-7 to *R*-11) of *R*-bicalutamide, and an isothiocyanate derivative of *S*-bicalutamide (*S*-6). Bicalutamide (*R*-1) is a known antiandrogen with stereoselective binding to the AR [11]. We determined

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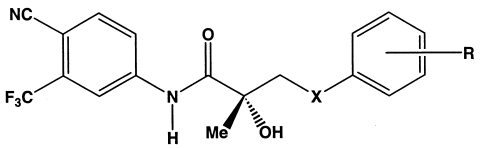
§ *Abbreviations*: AR, androgen receptor(s); MIB, mibolerone; PMSF, phenylmethylsulfonyl fluoride; and HAP, hydroxyapatite.

TABLE 1. K_i of synthesized affinity ligands



Compound	X	R	K_i* (nM)
R-1 (*R*-Bicalutamide)	SO_2	4-F	11 ± 2
R-2 (*R*-Thio-bicalutamide)	S	4-F	1.8 ± 0.5
R-3	SO_2	3-NCS	140 ± 10
R-4	SO_2	4-NCS	41 ± 2
R-5	S	3-NCS	240 ± 15
R-6	S	4-NCS	0.6 ± 0.1
R-7	S	3-$NCOCH_2Cl$	>1000
R-8	S	4-$NCOCH_2Cl$	1.7 ± 0.1
R-9	S	3-$NCOCH_2Br$	>1000
R-10	S	4-$NCOCH_2Br$	100 ± 10
R-11	SO_2	4-$NCOCH_2Br$	360 ± 30
R-12	S	3-NH_2	65 ± 25
R-13	S	4-NH_2	90 ± 15
S-6 (*S*-isomer)	S	4-NCS	430 ± 13

K_i values were determined from competitive binding studies of each ligand in the presence of 1 nM [^{3}H]MIB at 4°C for 18 hr. Values represent means ± SD of at least three experiments.

irreversible receptor inactivation by these chemoaffinity ligands using cytosolic AR from rat ventral prostate. Three isothiocyanate derivatives of *R*-bicalutamide (*R*-3, *R*-4, and *R*-6) were found to cause a significant reduction in AR binding sites for the highly specific AR ligand MIB. The data provide strong evidence that these isothiocyanate derivatives are the first reported chemoaffinity ligands for the AR.

MATERIALS AND METHODS

Materials

Commercially available *R*-proline and *S*-proline were purchased from Lancaster Synthesis. Methacryloylchloride and 3-chloroperbenzoic acid were obtained from Janssen Chimica. Thionyl chloride, 3-aminothiophenol, 4-aminothiophenol, peracetic acid, chloroacetyl chloride, and bromoacetyl bromide were purchased from the Aldrich Chemical Co. 4-Amino-2-trifluoromethylbenzonitrile was obtained from Lancaster Synthesis. [17α-*methyl*-^{3}H]Mibolerone ([^{3}H]MIB, 83.5 Ci/mmol) and unlabeled MIB were purchased from DuPont Research NEN Products. Triamcinolone acetonide, PMSF, Tris base, sodium molybdate, and dithiothreitol were purchased from the Sigma Chemical Co. HAP was obtained from Bio-Rad Laboratories. EcoLite^{TM} (+) scintillation fluid was purchased from ICN Research Products Division. All materials were used as received from the manufacturers.

Synthesis

The *R*-isomers of compounds *R*-1 to *R*-13 were prepared according to the general synthetic scheme reported for the enantiomers of bicalutamide, in particular for *R*-2 [11–13]. This synthetic route was proposed by Tucker and Chesterson [12] for the preparation of *S*-bicalutamide. We extended this approach successfully to preparation of the *R*-isomer of thio-bicalutamide, *R*-2, and bicalutamide, *R*-1 [11]. Detailed methods for synthesis of the affinity ligands described herein will be reported elsewhere.* Briefly, the reaction sequence started with coupling of the commercially available chiral auxiliary, *S*- or *R*-proline, with commercially available methacryloylchloride in aqueous acetone at 5–10° under Schottenn-Baumann conditions. *R*- or *S*-Prolineamide [(2*R*)- or (2*S*)-1-methacryloylpyrrolidine-2-carboxylic acid] formed at the first step was converted to its corresponding *R*- or *S*-bromolactone {(3*R*,8a*R*)- or (3*S*,8a*S*)-3-(bromomethyl)-3-methylperhydropyrrolo[2,1-*c*] [1, 4]oxazine-1,4-dione} using the Terashima method of asymmetric bromolactonization [14, 15]. This step was critical for the reaction sequence due to creation of the second asymmetric center. Hydrolysis of the bromolactone in 24% aqueous hydrobromic acid for 1 hr (instead of 8 hr in hydrochloric acid, as originally performed by Tucker *et al.* [13]) gave rise to *R*- or *S*-bromoacid [(2*R*)- or (2*S*)-3-bromo-2-hydroxy-2-methylpropanoic acid] in pure form, unlike the mixture of the corresponding bromo- and chloroacid (3-chloro-2-hydroxy-2-methylpropanoic acid) obtained in the procedure of Tucker *et al.* [13]. This bromoacid was a critical intermediate for all further synthetic manipulations in the present study.

Coupling of the chloroanhydride of the bromoacid (prepared *in situ* by reaction with thionyl chloride) with commercially available 4-amino-2-trifluoromethylbenzonitrile in dimethylacetamide at −10 to −15° gave rise to *R*- or *S*-bromoanilide {(4-[(2*R*)-3-bromo-2-hydroxy-2-methylpropanoyl]-2-(trifluoromethyl) benzonitrile) or (4-[(2*S*)-3-bromo-2-hydroxy-2- methylpropanoyl]-2-(trifluoromethyl)benzonitrile)}. The bromoanilides were coupled with commercially available 4-amino- or 3-aminothiophenol in tetrahydrofuran at room temperature. The final products of the reaction were the aniline derivatives *R*-12 and *R*-13 or *S*-13 (*S*-isomer of *R*-13), which then were used as the precursors for preparation of final affinity ligands.

Conversion of these aniline precursors *R*-12, and *R*-13 or *S*-13, to the isothiocyanate affinity ligands *R*-5, and *R*-6 or *S*-6, respectively, was achieved by their reaction with thiophosgene in the heterogeneous aqueous-organic mixture (ethyl acetate or methylene chloride with aqueous sodium bicarbonate solution) according to the procedure of Leclerc *et al.* [16]. Chloroacetyl and bromoacetyl compounds (*R*-7 to *R*-10) were prepared by acylation of the corresponding aniline precursors *R*-12 and *R*-13 using commercially available chloroacetyl chloride or bromoacetyl bromide in ethyl acetate or methylene chloride solution with solid calcium carbonate powder as a base. All thioester affinity ligands (*R*-5, *R*-6, and *R*-10) were oxidized

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to the corresponding sulfonyl derivatives (*R*-3, *R*-4, and *R*-11) by commercially available 3-chloroperbenzoic acid or peracetic acid in ethyl acetate or methylene chloride solutions. Structures of synthesized compounds were elucidated by ^{1}H- and ^{13}C-NMR, IR, and elemental analyses. All compounds were obtained at high purity. Stability of these compounds under incubation conditions for AR binding studies was determined by reversed phase high pressure liquid chromatography, and all compounds were found to be stable for the duration of incubation.

Buffers

Homogenization buffer consisted of 10 mM Tris, 1.5 mM disodium EDTA, 0.25 M sucrose, 10 mM sodium molybdate, and 1 mM PMSF and was adjusted to pH 7.4 [17]. Washing buffer was 50 mM Tris, pH 7.2.

Preparation of Cytosolic Androgen Receptor

Male Sprague-Dawley rats (Harlan Sprague-Dawley), weighing 248–290 g, were castrated 24 hr prior to the removal of prostates. Ventral prostates were removed surgically, weighed, and immersed immediately in ice-cold homogenization buffer. The prostate tissue (about 0.4 g per rat) was minced, weighed, and homogenized (PRO 200 homogenizer, PRO Scientific) with 1 mL of the homogenization buffer per 500 mg of prostate tissue. Then the homogenate was centrifuged at 105,000 *g* for 1 hr at 0° in a Beckman L8-M Ultracentrifuge (Beckman Instruments Inc.) [18]. The supernatant (cytosol) containing AR protein was removed and stored at −80° until used. Total protein was determined by Peterson's modification of the micro-Lowry method, using a commercially available protein assay kit from Sigma Diagnostics.

Assay of AR Binding Affinity

AR binding affinities of the synthesized ligands were determined by competitive binding in the presence of the high-affinity AR ligand [^{3}H]MIB. AR binding studies were performed by incubating increasing concentrations (10^{-3} to 10,000 nM) of each ligand with cytosol and a saturating concentration of [^{3}H]MIB (1 nM) at 4° for 18 hr. In preliminary experiments, the equilibrium dissociation constant (K_d) of MIB was determined under identical conditions by incubating increasing concentrations of [^{3}H]MIB (0.01 to 10 nM) with cytosol. We found that the minimum concentration of [^{3}H]MIB required to saturate AR sites in the cytosol preparation was 1 nM. Subsequent experiments used either 1 or 2 nM [^{3}H]MIB. The incubation mixtures also contained 1000 nM triamcinolone acetonide to block the interaction of MIB with progesterone receptors [19]. For the determination of nonspecific binding, separate experiments were conducted by adding 1000 nM MIB to the incubation mixture. Separation of bound and free radioactivity at the end of incubation was achieved by the HAP method, as described previously [11], and 0.8 mL of the ethanolic supernatant was added to 5 mL of scintillation fluid. Radioactivity was counted in a Beckman LS 6800 liquid scintillation counter (Beckman Instruments).

Exchange Assays

The potential of these ligands for covalent attachment to the AR was first examined using exchange assays. The exchange assay provided a rapid and less expensive method to screen for compounds capable of covalent attachment, as compared with Scatchard analyses. For exchange assays, cytosol was incubated with or without excess (10,000 nM) affinity label at 4° for 12 hr. Then AR were adsorbed onto HAP by addition of a slurry of HAP in 50 mM Tris and 1 mM KH_2PO_4 (pH 7.4). The tubes were incubated for 15 min at 4° with frequent vortexing, and HAP-bound AR were pelleted by centrifugation at 2500 *g* for 5 min (Speedfuge HSC10K, Savant Instruments Inc.). The pellet was washed three times with washing buffer to remove unbound affinity label. HAP-bound AR then were incubated with 2 nM [^{3}H]MIB and 1000 nM triamcinolone for 18 hr at 4° to determine the remaining [^{3}H]MIB binding sites. Nonspecific binding was determined by incubation of HAP-bound AR with 2 nM [^{3}H]MIB in the presence of 2000 nM MIB. Nonspecific binding was subtracted from total binding to determine specific binding of [^{3}H]MIB to AR. Cytosol preincubated without affinity label indicated the total number of available binding sites. Ligands that showed promising activity during exchange assays were examined further in competitive binding experiments.

Scatchard Analysis

Cytosol was incubated with increasing concentrations of [^{3}H]MIB (0.1 to 1 nM) and an affinity label (1 to 10,000 nM) in the same tube at 4° for 48 hr. A longer incubation time (48 hr) was used to ensure equilibrium binding at all concentrations of [^{3}H]MIB and ligand. In studies in our laboratory, we found that maximal specific binding of [^{3}H]MIB to cytosolic AR was achieved within 18 hr and remained at the maximal level for at least 96 hr. As compared with exchange assays, competitive binding experiments offer the advantage of ascertaining the nature and specificity of ligand interaction. The specificity of AR interaction was determined by measurement of nonspecific binding in the presence of excess unlabeled MIB. Bound and free radioactivity were separated by the HAP method. Scatchard analysis of the specific binding data were used to determine the reduction in AR sites.

Data Analysis

Specific binding for each experiment was calculated by subtracting the binding of [^{3}H]MIB observed in the presence of excess unlabeled MIB (nonspecific binding) from the binding of [^{3}H]MIB observed in the absence of unla-

beled MIB (total binding). Competitive radioligand displacement curves then were constructed with percent specific binding (specific binding of [^{3}H]MIB at a particular ligand concentration expressed as a percentage of the specific binding of [^{3}H]MIB in the absence of ligand) on the vertical axis and ligand concentration on the horizontal axis. The ligand concentration that reduced the percentage of specific binding by 50% (IC_{50}) was determined by computer fitting of data for the competitive binding of each AR ligand to the following equation, using a Fortran subroutine written for PCNONLIN (SCI Software):

$$B = B_O\left[1 - \frac{C}{IC_{50} + C}\right],$$

where B is the specific binding of [^{3}H]MIB in the presence of a particular concentration of ligand, B_o is the specific binding of [^{3}H]MIB in the absence of ligand, and C is the ligand concentration. Binding affinity of the ligand then was compared using the equilibrium dissociation constant of the inhibitor (K_i). The K_i of each ligand was calculated using the equation:

$$K_i = \frac{IC_{50} \cdot K_d}{L + K_d},$$

where K_d is the equilibrium dissociation constant of [^{3}H]MIB, and L is the concentration of [^{3}H]MIB (1 nM). Data from Scatchard experiments were analyzed using a modified form of the Scatchard equation:

$$B/F = \left[\frac{-1}{K_d}\right] \cdot B + \frac{B_{max}}{K_d},$$

where B is the concentration of radioligand ([^{3}H]MIB) present as ligand–AR complex, F is the concentration of free [^{3}H]MIB in the incubation mixture, and B_{max} is the maximum concentration of [^{3}H]MIB bound (picomoles per gram of total protein), which is also the concentration of available AR binding sites in the incubation mixture. All statistical comparisons were performed at a 5% level of significance.

RESULTS

AR Binding Affinity

The K_d for the binding of [^{3}H]MIB to the AR, as determined by Scatchard analysis, was 0.19 ± 0.01 nM. The synthesized compounds showed a wide range of binding affinities for the AR (Table 1). We previously reported that *R*-bicalutamide has a 30-fold higher AR binding affinity than its *S*-isomer [11]. The *S*-isomers synthesized in these studies also had significantly lower binding affinities than the *R*-isomers (only the K_i value of *S*-6 is shown). Further, thioesters and *para*-substituted analogs demonstrated greater AR binding affinities than the corresponding sulfonyl and *meta*-substituted analogs, respectively. The only exception was the *m*-isothiocyanate derivative (*R*-3), which had a higher affinity than the thio-*m*-isothiocyanate (*R*-5). The *p*-isothiocyanate derivative (*R*-4) (K_i = 41 ± 2 nM) had about three times the affinity of the *meta* derivative (*R*-3) (K_i = 140 ± 10 nM), whereas the thio-*p*-isothiocyanate derivative (*R*-6) bound the AR with extremely high affinity (K_i = 0.6 ± 0.1 nM). However, it is important to note that these isothiocyanate derivatives reacted with the AR irreversibly (discussed below). Thus, the K_i values for these ligands should be considered "apparent K_i" values, denoting their interaction via both reversible and irreversible means. The thio-*p*-chloroacetamide derivative (*R*-8, K_i = 1.7 ± 0.1 nM) was also a high-affinity compound, whereas the *meta* derivative (*R*-7) had a very low AR binding affinity. The AR binding affinities of the thio-*p*-isothiocyanate (*R*-6) and thio-*p*-chloroacetamide (*R*-8) were comparable to that of testosterone (K_i = 1.1 ± 0.2 nM), and this was a key finding in our recent discovery of androgens [20].

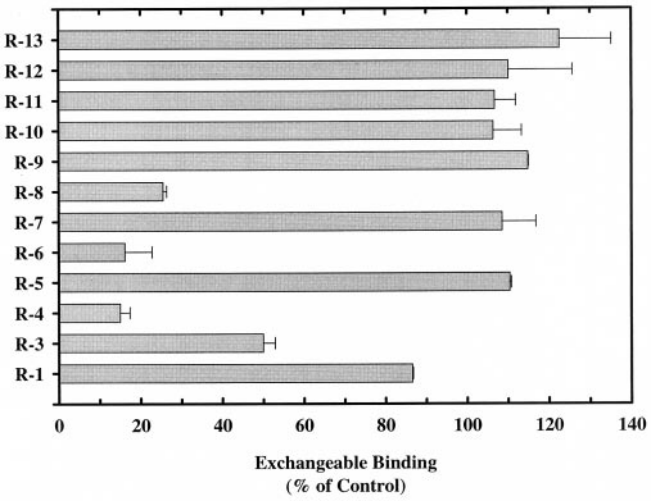

FIG. 1. Exchangeable specific binding of [^{3}H]MIB after incubation with ligand. Cytosol was incubated with a 10,000 nM concentration of each ligand for 12 hr at 4°. AR were adsorbed with HAP, and HAP-bound AR were incubated with 2 nM [^{3}H]MIB in the presence of 1000 nM triamcinolone at 4° for 18 hr. Values are expressed as mean (±SD) percent of specific [^{3}H]MIB binding to cytosol preincubated without ligand. *R*-Bicalutamide and aniline derivatives were used as reversible controls. Exchangeable specific binding of [^{3}H]MIB in the absence of any competitor was 2270 ± 267 dpm. Experiments were performed in triplicate for each ligand.

Exchange Assays

Exchange assays were used to identify potential affinity ligands from among the synthesized AR ligands (Fig. 1). AR were preincubated with an excess of ligand to ensure favorable conditions for covalent attachment even for affinity labels with low binding affinities. For a reversible ligand, recovery of specific binding sites by [^{3}H]MIB was expected to be near 100%. A significant decrease in exchangeable binding of [^{3}H]MIB would indicate receptor

inactivation, suggesting covalent attachment of the affinity label and identifying it as a promising candidate for more in-depth analysis using competitive binding experiments (i.e. Scatchard analysis). We used *R*-bicalutamide (*R*-1) and the aniline derivatives (*R*-12 and *R*-13) as controls for reversible binding. The recovery of exchangeable binding sites by 1 nM [^{3}H]MIB after incubation with these ligands was 86 ± 1% (*R*-1), 110 ± 15% (*R*-12), and 123 ± 12% (*R*-13), respectively, demonstrating the ability of the exchange assay to rule out reversible ligands (Fig. 1). The *m*-isothiocyanate (*R*-3), *p*-isothiocyanate (*R*-4), thio-*p*-isothiocyanate (*R*-6), and thio-*p*-chloroacetamide (*R*-8) derivatives reduced exchangeable specific binding to 50 ± 3, 15 ± 2, 16 ± 7, and 26 ± 1%, respectively, suggesting that these ligands interacted in a specific and irreversible manner with the AR (Fig. 1). Several lines of evidence support this assertion, as discussed later. In a separate experiment, the effects of the *R*- and *S*-isomers of thio-*p*-isothiocyanate (*R*-6 and *S*-6, respectively), at 2000 nM each, were determined. *R*-Thio-*p*-isothiocyanate (*R*-6) reduced specific [^{3}H]MIB binding to 14 ± 7%, whereas exchangeable specific binding after incubation with *S*-thio-*p*-isothiocyanate (*S*-6) was 99 ± 11%. The bromoacetamide derivatives (*R*-9, *R*-10, and *R*-11), which have very low specificity of labeling [1], had no effect on the exchangeable specific binding of [^{3}H]MIB. The thio-*m*-isothiocyanate derivative (*R*-5) also did not reduce exchangeable specific binding of [^{3}H]MIB, compared with controls incubated without ligand. Only the ligands that showed significant reduction in specific binding of [^{3}H]MIB in the exchange assays were investigated further, using competitive binding assays (Scatchard analysis).

Scatchard Analysis of AR Binding

To further examine the nature and specificity of interaction of *R*-3, *R*-4, *R*-6, and *R*-8, we performed competitive binding experiments. In these experiments, lower concentrations of the affinity labels were used. *R*-Bicalutamide and unlabeled MIB were included as reversible controls. We exposed the AR to the affinity label along with a range of [^{3}H]MIB concentrations, and estimated B_{max}, the concentration of AR binding sites in the incubation mixtures, using Scatchard analysis (Fig. 2). These experiments were repeated with different concentrations of each affinity label to show progressive reduction in B_{max} with increasing ligand concentration. In Scatchard analysis, the presence of a reversible ligand increases the apparent K_d of radioligand ([^{3}H]MIB) binding, without affecting B_{max}. An irreversible ligand, however, is expected to inactivate receptor binding sites, thereby reducing B_{max} with increasing ligand concentration, until maximal inactivation is achieved. A purely irreversible ligand has no effect on the K_d of radioligand binding. However, in affinity labeling, specificity is achieved by the initial reversible interaction of the affinity label with the AR, followed by covalent attachment at a nucleophilic site in the ligand binding pocket [1, 2]. In the synthesis of the *R*-bicalutamide analogs, the tertiary hydroxy group, thought to be the site of reversible interaction with the AR [13, 21], was preserved. The potential AR affinity labels, therefore, were expected first to interact reversibly with the AR, and then to label covalently. The presence of an affinity label, therefore, would result in an increase in the apparent K_d of [^{3}H]MIB binding, along with a decrease in B_{max}.

At *R*-bicalutamide (*R*-1) concentrations of 50, 100, and 200 nM, the apparent K_d of [^{3}H]MIB binding progressively increased, as evidenced by a decrease in the slopes of the lines representing [^{3}H]MIB binding at each ligand concentration. However, as would be expected, there was no reduction in B_{max} (x-intercept) (Fig. 2, top panel). Similarly, in the presence of 1 nM MIB, there was an increase in the apparent K_d of [^{3}H]MIB binding, but no change in B_{max} occurred (Fig. 2, top panel).

The thio-*p*-isothiocyanate (*R*-6) derivative caused progressive and significant reduction in B_{max} at increasing concentrations (Fig. 2, bottom, left panel). In incubation mixtures containing 250 nM thio-*p*-isothiocyanate (*R*-6), the concentration of binding sites was only 50% that in incubation mixtures without ligand. At ligand concentrations of 500 and 1000 nM, B_{max} further decreased to 32 and 29%, respectively. These results compare well with the results of the exchange assay in terms of the labeling efficiency of this ligand. The exchange assay showed that at 10,000 nM thio-*p*-isothiocyanate (*R*-6), AR binding sites decreased to 16% of total. These results confirm that the thio-*p*-isothiocyanate (*R*-6) derivative bound irreversibly to the AR.

The *p*-isothiocyanate derivative (*R*-4) and the *m*-isothiocyanate derivative (*R*-3) also caused progressive and significant reduction in B_{max} at increasing concentrations. The *p*-isothiocyanate derivative (*R*-4), at concentrations of 50, 250, 500, and 5000 nM, decreased B_{max} values to 87, 44, 42, and 13%, respectively, of those for incubation mixtures without ligand (Fig. 2, middle, right panel). In the presence of 2,000, 5,000, and 10,000 nM concentrations of the *m*-isothiocyanate derivative (*R*-3), B_{max} values were 89, 74, and 42%, respectively, of incubation mixtures without ligand (Fig. 2, middle, left panel). The results of the Scatchard analysis for both the *p*-isothiocyanate (*R*-4) and the *m*-isothiocyanate (*R*-3) derivatives also compared well with the results of the exchange assay.

The thio-*p*-chloroacetamide (*R*-8) derivative caused a progressive increase in the apparent K_d of [^{3}H]MIB at concentrations of 20 and 50 nM, but did not show a significant decrease in B_{max}, indicating that it is a reversible ligand (Fig. 2, bottom, right panel). This contradiction with the result of the exchange assay is explained by the high binding affinity of the thio-*p*-chloroacetamide derivative (*R*-8). Ligands with low dissociation constants dissociate at a slow rate from the AR, and, therefore, are not displaced completely by [^{3}H]MIB within the period of incubation in the exchange assay. This was evident from the effect of preincubation with 1000 nM unlabeled MIB,

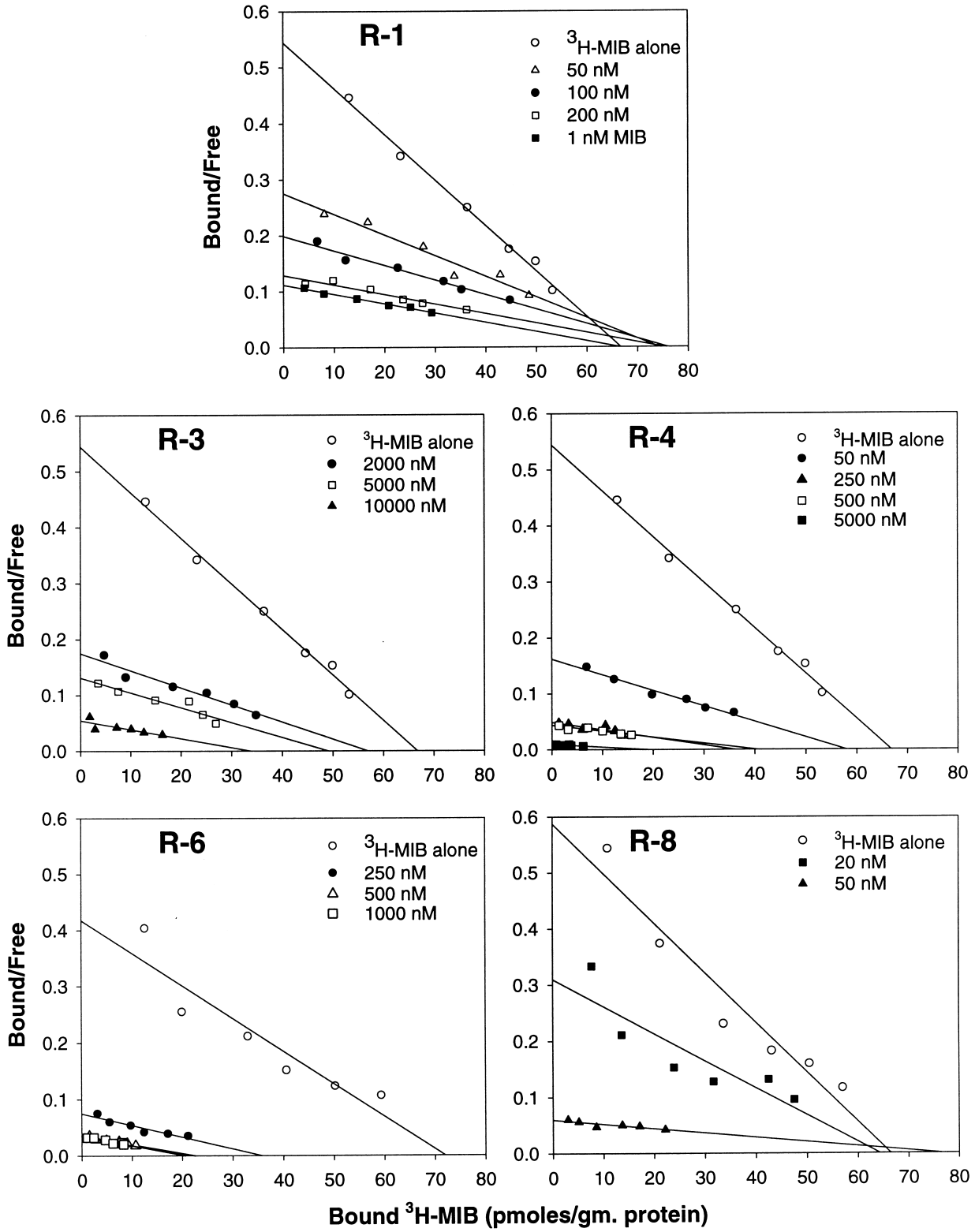

FIG. 2. Scatchard plots of reversible and irreversible AR binding. Increasing concentrations (0.1, 0.2, 0.4, 0.6, 0.8, and 1 nM) of [^{3}H]MIB were incubated without ligand, or with the indicated concentrations of various ligands. Incubations were performed at 4° for 48 hr. Lines represent linear least squares regression of the experimental data. Symbols represent mean binding data (N = 2).

which has 10 times the affinity of *R*-8, in an exchange assay (Fig. 3). Exchangeable specific binding was 21 ± 2% after an 18-hr incubation with [^{3}H]MIB, and increased to 29 ± 2 and 58 ± 1% after 48 and 90 hr, respectively, indicating slow displacement of bound MIB. For the irreversible ligand, *R*-thio-*p*-isothiocyanate (*R*-6), exchangeable specific binding was 14 ± 7, 13 ± 2, and 15 ± 2%, respectively, after 18-, 48-, and 90-hr incubations with

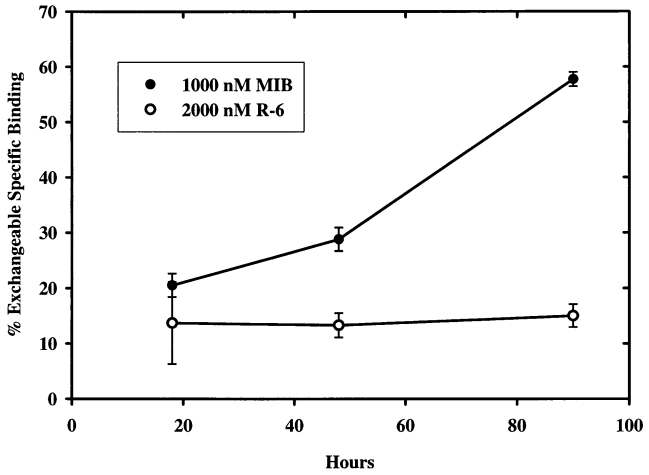

FIG. 3. Recovery of exchangeable specific binding sites after incubation with a high-affinity reversible ligand and an affinity label. Cytosol was incubated with either 1000 nM MIB or 2000 nM *R*-6 for 1 hr at 4°. AR were adsorbed with HAP, and incubated for 18, 48, and 90 hr with 2 nM [^{3}H]MIB, and 1000 nM triamcinolone, at 4°. Nonspecific binding was determined by including 2000 nM MIB in the incubation mixture. Exchangeable specific binding of [^{3}H]MIB in the absence of any competitor was 2270 ± 267 dpm. Data represent means ± SD of triplicate determinations.

[^{3}H]MIB. Exchangeable specific binding of [^{3}H]MIB, therefore, did not increase with longer incubation times, indicating true irreversible binding of *R*-6 to the AR.

We were able to distinguish between high-affinity reversible and irreversible binding, using Scatchard analysis. MIB did not show apparent irreversibility due to high affinity in the Scatchard analysis (Fig. 2, top panel). The results of the Scatchard analysis, therefore, confirmed that the thio-*p*-chloroacetamide (*R*-8) derivative was a reversible ligand. The results also provide strong evidence that the thio-*p*-isothiocyanate (*R*-6), the *p*-isothiocyanate (*R*-4), and the *m*-isothiocyanate (*R*-3) derivatives of *R*-bicalutamide are the first chemoaffinity ligands for the AR.

DISCUSSION

The [^{3}H]MIB binding assay has been used extensively for the measurement of AR levels in various tissues [19, 22–24]. In the rat prostate, MIB was shown to bind specifically to AR, and this binding was not competed for by progestins, estrogens, or glucocorticoids [22]. Although MIB was found to bind progesterone receptors in other species, this binding was blocked completely by an excess of triamcinolone acetonide [19]. In this report, Scatchard analyses of [^{3}H]MIB binding to rat ventral prostate cytosol indicated a single class of high-affinity, low-capacity binding sites. Exchangeable [^{3}H]MIB binding is, therefore, a specific and accurate measure of functional AR sites. The ligands in this series were derived from the nonsteroidal antiandrogen *R*-bicalutamide, which is a specific AR ligand and is devoid of progestational and glucocorticoid activities [25].

Several lines of evidence indicate specific and efficient irreversible binding of these novel affinity labels to the AR. Besides covalent attachment, two alternative explanations for the observed decreases in exchangeable specific binding of [^{3}H]MIB after treatment with *R*-3, *R*-4, *R*-6, and *R*-8 were possible: (i) nonspecific denaturation of the AR, and (ii) apparent irreversibility due to high-affinity binding. Nonspecific denaturation of the AR due to high ligand concentrations can be ruled out. The *S*-isomer (*S*-6) of the most potent affinity label, the *R*-thio-*p*-isothiocyanate derivative (*R*-6), was synthesized and assayed to provide convincing evidence of both AR specificity and absence of nonspecific denaturing effects on the AR. *S*-Thio-*p*-isothiocyanate (*S*-6) (K_i = 430 ± 13 nM) showed a considerably lower AR binding affinity than the *R*-isomer (*R*-6) (K_i = 0.6 ± 0.1 nM), which was in agreement with the enantioselective differences in AR binding of bicalutamide and its derivatives observed in our laboratory [11]. The lack of initial specific, reversible interaction with the AR prevented covalent labeling by the *S*-isomer, whereas at the same concentration (2000 nM), the *R*-isomer inactivated more than 80% of the AR sites. Since the two isomers have the same chemical reactivities with non-chiral reagents, these results indicate that *R*-6 labeled the AR specifically and did not have significant nonspecific denaturing effects on the AR. Nonspecific effects are not expected to be enantioselective. Further, the thio-*m*-isothiocyanate (*R*-5) derivative, which bears the same electrophilic functional group as *R*-3, *R*-4, and *R*-6, had no effect on exchangeable specific binding, due to a significantly lower AR binding affinity. The majority of compounds in this series, which are close structural analogs, had no effect on exchangeable specific binding of [^{3}H]MIB at the high concentration (10,000 nM) used in the exchange assay, indicating that nonspecific destruction of the AR does not contribute significantly to the observed decrease in exchangeable specific binding for isothiocyanate derivatives.

Studies with reversible controls provided sufficient evidence that the binding of *R*-3, *R*-4, and *R*-6 was true irreversible binding, and not apparent irreversibility due to high AR binding affinity. The *m*-isothiocyanate (*R*-3) and *p*-isothiocyanate (*R*-4) derivatives had apparent AR binding affinities that were about 12- and 4-fold lower than *R*-bicalutamide (Table 1). Exchangeable specific binding sites recovered after incubation with *R*-3 and *R*-4 were significantly lower than those recovered after incubation with *R*-bicalutamide, suggesting that *R*-3 and *R*-4 bound the AR covalently. The high-affinity reversible ligand MIB showed apparent irreversibility after 18 hr of incubation with [^{3}H]MIB. However, when MIB-bound AR were incubated with [^{3}H]MIB for longer periods of time, an increasing number of AR sites were recovered, indicating slow dissociation of MIB (Fig. 3). In the same experiment, exchangeable specific binding after incubation with *R*-6 did not increase upon longer periods of incubation with [^{3}H]MIB, indicating that *R*-6 bound the AR efficiently and irreversibly. Irreversible binding of the isothiocyanate de-

rivatives, *R*-3, *R*-4, and *R*-6, was confirmed further by competitive binding studies (Scatchard plots). The reversible controls, *R*-bicalutamide and MIB, did not show AR inactivation in these studies, whereas the isothiocyanate derivatives showed a reduction in B_{max} at concentrations lower than those used in the exchange assay, indicating specific inactivation of AR sites by these ligands.

Analyses of AR binding affinities and theoretical chemical reactivities of affinity labels provide more evidence that the isothiocyanate derivatives, *R*-3, *R*-4, and *R*-6, interact specifically and irreversibly with the AR. Scatchard analyses showed that the labeling efficiencies of the isothiocyanate derivatives, *R*-3, *R*-4, and *R*-6, in terms of the extent of reduction in B_{max}, increased with increasing AR binding affinities of these ligands. Derivatives containing bromoacetamide, which is a reactive group with very low specificity [1], did not show AR inactivation in the exchange assays. The chloroacetamide derivative, *R*-8, also was found to be reversible by Scatchard analysis. Generally, it is accepted that haloacetamides have lower specificity than isothiocyanates, which have high specificity for amino groups. Our results therefore, show that efficiency of labeling correlates with AR binding affinity and not with the reactivity of the functional groups, suggesting that irreversible binding of the isothiocyanate derivatives occurs after initial specific, reversible association with the AR. The thio-*m*-isothiocyanate (*R*-5) and the *S*-thio-*p*-isothiocyanate (*S*-6) derivatives did not show irreversible binding due to their low AR binding affinity and lack of initial reversible interaction with the AR. The inability of *R*-5 and *S*-6 to inactivate the AR further speaks to the stereochemical and structural elements required for AR specificity.

These studies provide strong evidence that the isothiocyanate derivatives *R*-3, *R*-4, and *R*-6 label the AR specifically and covalently and represent the first affinity labels for the AR. Derivative *R*-6 has especially high labeling efficiency, and is also specific. Previously, radiolabeled forms of affinity labels were used to identify the specific amino acids in the estrogen receptor and glucocorticoid receptor involved in covalent ligand binding [26, 27]. Further work is being done in our laboratory to synthesize radiolabeled forms of the isothiocyanate derivatives to identify the specific amino acid(s) involved in attachment.

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