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Discovery of Nonsteroidal Androgens

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Nonsteroidal androgens have not been reported. During studies to identify affinity ligands for the androgen receptor in our laboratory, we synthesized several electrophilic nonsteroidal ligands for the androgen receptor and examined their receptor binding affinity and ability to stimulate receptor-mediated transcriptional activation. We found that three of these ligands (1) bound the androgen receptor with affinity similar to that of dihydrotestosterone (the endogenous ligand) and (2) mimicked the effects of dihydrotestosterone on receptor-mediated transcriptional activation (i.e., they were receptor agonists). These studies demonstrate that nonsteroidal ligands can be structurally modified to produce agonist activity. These ligands thus represent the first members of a novel class of androgens with potential therapeutic applications in male fertility and hormone replacement therapy.

Androgenic steroids play an important role in many physiologic processes, including the development and maintenance of male sexual characteristics such as muscle and bone mass, prostate growth, spermatogenesis, and the male hair pattern (1). Testosterone is the principal steroid secreted by the testes and is the primary circulating androgen found in the plasma of males. Testosterone is converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase in many peripheral tissues. DHT is thus thought to serve as the intracellular mediator for most androgen actions (2).

Exogenous steroidal ligands which bind to the AR and act as androgens (e.g. testosterone enanthate) or as antiandrogens (e.g. cyproterone acetate) have been known for many years and are used clinically (3). Although nonsteroidal antiandrogens are used for the treatment of hormone-dependent prostate cancer (4), nonsteroidal androgens have not been reported. Nonsteroidal androgens would have clinical utility for aging or hypogonadal men requiring hormone replacement therapy and as regulators of male fertility. Moreover, based on the known properties of nonsteroidal antiandrogens (5, 6), nonsteroidal androgens would likely avoid many of the undesirable physicochemical and pharmacokinetic properties of their steroidal counterparts, including poor oral bioavailability and rapid hepatic metabolism.

During studies in our laboratory to search for electrophilic affinity ligands for the AR, we discovered that structural modification of known nonsteroidal antiandrogens resulted in androgenic activity. These compounds represent a new class of androgenic drugs, and are the first nonsteroidal agonists for the AR. The results of our studies to examine the in vitro biological activity of these nonsteroidal androgens are described herein (Fig. 1).

MATERIALS AND METHODS

Chemicals. (17α-methyl-^{3}H)-Mibolerone (^{3}H-MIB, 83.5 Ci/mmol) and unlabeled MIB were purchased from DuPont Research NEN Products (Boston, MA). Triamcinolone acetonide, phenylmethylsulfonyl fluoride (PMSF), TRIS base, sodium molybdate and dithiothreitol were purchased from Sigma Chemical Company (St. Louis, MO). Hydroxyapatite (HAP) was purchased from BIO-RAD Laboratories (Hercules, CA). EcoLite Plus™ scintillation cocktail was purchased from ICN Research Products Division (Costa Mesa, CA). Dulbecco's modified essential medium (DMEM) and Lipofectamine™ transfection reagent were purchased from Life Technologies (Gaithersburg, MD). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals, Inc. (Norcross, GA). All materials were used as received from the manufacturer.

Organic synthesis. R-isomers of compounds **R-1** to **R-5** were prepared according to the general synthetic scheme reported for the enantiomers of bicalutamide using commercially available R-proline (Lancaster Synthesis, Wyndham, NH) as the chiral auxillary (7, 8). S-isomers were prepared using S-proline as chiral auxillary. The structures of synthesized compounds were in accordance with the elemental analyses and spectroscopic data (^{1}H and ^{13}C NMR, and IR). Detailed syntheses of the above mentioned compounds were presented (9) and will be the subject of future communications.

Preparation of cytosolic androgen receptor. Male Sprague-Dawley rats (Harlan Biosciences, Indianapolis, IN), weighing approximately 250 g, were castrated 24 hours prior to the removal of prostates.

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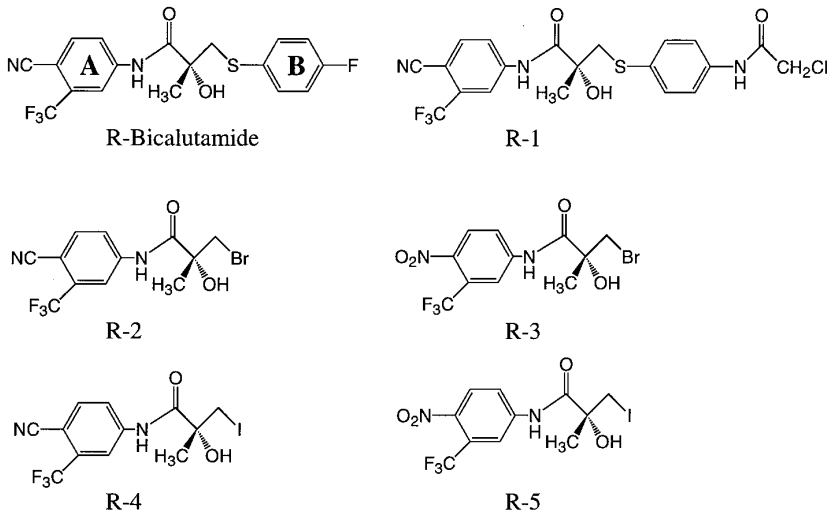

FIG. 1. Structure of nonsteroidal androgen receptor ligands. Aromatic rings in R-bicalutamide are designated A and B to clarify discussion of structural modifications in the text.

Ventral prostates were surgically removed, weighed, and immersed immediately in an ice-cold homogenization buffer consisting of 10 mM TRIS, 1.5 mM disodium EDTA, 0.5 mM dithiothreitol, 0.25 M sucrose, 10 mM sodium molybdate, and 1 mM PMSF adjusted to pH 7.2. The prostate tissue (about 0.4 g per rat) was minced, weighed, and homogenized (Model PRO 200 homogenizer, PRO Scientific, Monroe, CT) with 1 mL of the homogenization buffer per 500 mg of prostate tissue. The homogenate was then centrifuged at 114,000 g for 1 hour at 0°C in an ultracentrifuge (Model L8-M, Beckman Instruments Inc., Palo Alto, CA). The supernatant (cytosol) containing AR protein was removed and stored at −80°C until use.

Competitive binding studies. AR binding affinities of the synthesized ligands were determined by competitive binding in the presence of the high affinity AR ligand, ^{3}H-MIB. In preliminary experiments, the equilibrium dissociation constant (Kd) of MIB was determined at 4°C for 18 hours 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 to saturate AR sites in the cytosol preparation was 1 nM. AR binding studies for the ligands of interest were then performed under identical conditions by incubating increasing concentrations (10^{-3} nM to 10,000 nM) of each ligand with cytosol and a saturating concentration of ^{3}H-MIB (1 nM). The incubate also contained 1 μM triamcinolone to prevent interaction of ^{3}H-MIB with glucocorticoid and progesterone receptors (10). For the determination of non-specific binding, separate experiments were conducted by adding 1,000 nM MIB to the incubate. All experiments were repeated three times or more. Separation of bound and free radioactivity at the end of incubation was achieved by the HAP method, as previously described (7), and 0.8 mL of the ethanolic supernatant was added to 5 mL of scintillation cocktail. Radioactivity was counted in a liquid scintillation counter (Model LS6800, Beckman Instruments Inc., Palo Alto, CA).

Data analysis of AR binding affinity. To determine the Kd of MIB, data were analyzed using a modified form of the Scatchard equation:

$$B/F = \left[\frac{-1}{Kd}\right] * B + \frac{Bmax}{Kd} \qquad [1]$$

where B was the concentration of specifically-bound ^{3}H-MIB in the incubate, F was the concentration of free ^{3}H-MIB in the incubate, and Bmax was the maximum concentration of ^{3}H-MIB specifically bound to AR, which is also the concentration of available AR binding sites in the incubate. Experimental data were computer-fit to equations using fortran subroutines written for PCNONLIN (SCI Software, Lexington, KY).

For competition binding experiments, competitive displacement curves were constructed for each ligand 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 the data for each ligand to the following equation,

$$B = BO\left\{1 - \frac{C}{IC_{50} + C}\right\} \qquad [2]$$

where B0 was the specific binding of ^{3}H-MIB in the absence of ligand, and C was the ligand concentration. Binding affinities of the ligands were then compared using the equilibrium dissociation constant (Ki) of each ligand, as calculated using equation 3:

$$Ki = \frac{IC_{50} * Kd}{L + Kd} \qquad [3]$$

where Kd was the equilibrium dissociation constant of ^{3}H-MIB as determined in equation 1, and L was the concentration of ^{3}H-MIB in the incubate (i.e., 1 nM).

Transcriptional activation. The agonist activity of each ligand was examined using transfected CV-1 cells (American Type Culture Collection, Rockville, MD). CV-1 cells were maintained in DMEM containing 10% FBS at 37°C in a humidified atmosphere containing 5% carbon dioxide. DMEM without phenol red was used during transfection studies. One day before transfection, monolayer cells were removed from flasks and plated at a density of 4.5×10^5 cells/well in 6-well tissue culture plates. Cells in each well were then co-transfected with 100 ng of an AR expression vector (plasmid pCMVhAR; generously provided by Dr. Elizabeth Wilson at The University of North Carolina, Chapel Hill, NC), 2.5 μg of a luciferase reporter vector (plasmid pMMTV-Luc; generously provided by Dr.

TABLE 1

Mean (±S.D.) Binding Affinity and Transcriptional Activation of Nonsteroidal Ligands

Ligand	Ki (nM)	Efficacy[a] (% of DHT)	Potency[b] (nM)
DHT	0.28 ± 0.02	100[d]	1 nM
R-Bicalutamide	11.0 ± 1.5[c]	8.28 ± 2.66	1,000 nM
R-1	1.65 ± 0.10	97.5 ± 21.6	100 nM
R-2	7.98 ± 0.86	18.5 ± 5.08	500 nM
R-3	0.30 ± 0.12	82.9 ± 14.0	500 nM
R-4	2.79 ± 1.35	20.5 ± 7.57	500 nM
R-5	0.86 ± 0.11	88.6 ± 18.0	500 nM

[a] Maximal percentage of transcriptional activation observed for each ligand.

[b] The lowest concentration of the ligand capable of maximally stimulating AR-mediated transcription during transfection experiments.

[c] Reference 7.

[d] Ratio of luciferase activity to β-galactosidase activity was 397 ± 144 in the absence of any ligand (control) and 145,000 ± 33,000 in the presence of 1 nM DHT.

Ronald Evans at The Salk Institute, San Diego, CA), and 2.5 μg of a control β-galactosidase vector (plasmid pSV-β-galactosidase; Promega Corporation, Madison, WI). Transfection was performed using Lipofectamine according to the manufacturer's instructions. Ten hours after adding the transfection reagent, cells were washed twice with DMEM containing 0.2% FBS and then returned to the incubator. After an additional 10 to 12 hours of incubation, the medium was removed and replaced with DMEM containing 0.2% FBS and various concentrations of DHT or the ligand of interest. Final ligand concentrations were 0.1, 1, 10, 50, 100, 500 and 1,000 nM. The drug-containing medium was replaced after 24 hours of incubation to minimize the possible effects of ligand degradation on our measurement of transcriptional activation. Control studies in which neither DHT nor ligand were added to the medium were also performed. Cells were washed with phosphate buffered saline, pH 7.4, after 48 hours of drug treatment and harvested using 350 μL of reporter lysis buffer (Promega Corporation, Madison, WI). Cell lysates were transferred to a microcentrifuge tube, vortexed briefly, and centrifuged at 12,000g for 2 minutes. An aliquot (150 μL) of supernatant was used for determination of β-galactosidase activity in each well using a spectrophotometer (Cary Model 1E, Varian Associates, Sunnyvale, CA) set at a wavelength of 420 nm. A separate aliquot (100 μL) was used for determination of luciferase activity assay (Luciferase Assay System, Promega Corporation, Madison, WI) using an automated luminometer (Model AutoLumat LB953, Wallac Inc., Gaithersburg, MD). Viable cell numbers and transfection efficiency in each well were normalized by expressing the transcriptional activation as a ratio of the luciferase activity to β-galactosidase activity in individual wells. The efficacy of individual compounds compared to DHT was then calculated by dividing (the maximal transcriptional activation observed for each ligand) by (the maximal transcriptional activation observed for DHT). All experiments were performed in triplicate. Potency was reported as the lowest concentration of the ligand used during transfection experiments capable of producing maximal AR-mediated transcriptional activation.

RESULTS AND DISCUSSION

The Kd for ^{3}H-MIB in our studies (0.19 ± 0.01 nM; mean ± S.D., n=3) and the binding affinity of DHT (Table 1) were similar to previously reported values (7, 11). The Ki of R-bicalutamide was also similar to that previously reported, and was consistent with the binding affinity of other known nonsteroidal antiandrogens (4, 7, 8). Surprisingly, several R-isomers of these analogs bound the AR with affinity similar to that of DHT, and with much greater affinity than R-bicalutamide (Table 1). Substitution with a chloroacetamido (**R-1**) group at the para-position of aromatic ring B resulted in a significant increase in AR binding affinity. Likewise, ligands lacking the sulfonyl moiety and ring B (**R-2** to **R-5**) bound the AR with high affinity. Further, it is important to note that this affinity could be increased by replacing the para-cyano moiety in ring A with a nitro- functional group, suggesting that hydrogen bonding at this position may be critical to interaction with the AR. Further structural modifications to introduce the para-nitro substituent in ligands maintaining both aromatic rings (e.g. **R-1**) may therefore prove useful in identifying additional lead compounds, and are ongoing in our laboratory. The AR binding affinity (Ki value) of S-isomers was at least 10-fold greater than the Ki values for R-isomers in all cases (not shown). Thus, only the R-isomers of the synthesized analogs were evaluated for their ability to stimulate AR-mediated transcriptional activation.

In all cases, AR-mediated transcriptional activation increased with increasing ligand concentrations and then plateaued at higher concentrations. However, the efficacy (i.e., maximal degree of AR-mediated transcriptional activation observed) and potency (i.e., the lowest concentration of ligand capable of inducing maximal transcriptional activation) differed greatly between ligands (Table 1). For example, R-bicalutamide, a known antiandrogen, was unable to stimulate AR-mediated transcriptional activation at even the highest concentrations tested (i.e., mean efficacy of 8.3% at 1,000 nM). Similarly, compounds **R-2** and **R-4** which lack the sulfonyl moiety and ring B produced only minimal (<20%) efficacy. As noted in our studies of AR binding affinity, substitution of a para-nitro functional group in ring A significantly increased the degree of efficacy elicited by this series of ligands (e.g. compare **R-2** to **R-3** and **R-4** to **R-5**). However, the potency of these compounds was approximately 500-fold lower than that observed with DHT. Structural modifications in ring B to include the chloroacetamido- functional group at the para-position (ligand **R-1**) resulted in the most potent agonist activity. Ligand R-1 demonstrated a mean efficacy of approximately 98% at concentrations as low as 100 nM. Despite the electrophilic character of the chloroacetamido- substituent, detailed studies in our laboratories have shown that this ligand does not covalently bind the AR (12). Nevertheless, we recently initiated studies to synthesize and examine a ligand incorporating an acetamido- functional group at this position, as a means to explore the possibility of non-electrophilic analogs. It is clear that additional structural modification

of these ligands (e.g. incorporation of a para-nitro functional group in ring A of compound **R-1**) to improve the efficacy and potency of androgenic activity would be a worthwhile exercise.

CONCLUSIONS

These studies demonstrate that nonsteroidal ligands can be structurally modified to produce agonist activity. These ligands thus represent the first members of a novel class of androgens with potential therapeutic applications in male fertility and hormone replacement therapy. Additional efforts for drug discovery in this important area are needed and will be the subject of future research in our laboratory.

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REFERENCES

1. Matsumoto, A. M. (1994) *Endocrinol. Met. Clin. N. Am.* **23,** 857–875.
2. Zhou, Z. X., Lane, M. V., Kemppainen, J. A., French, F. S., and Wilson, E. M. (1995) *Molec. Endocrinol.* **9,** 208–218.
3. Wu, F. C. W. (1988) *Clin. Endocrinol.* **29,** 443–465.
4. Teutsch, G., Goubet, F., Battman, T., Bonfils, A., Bouchoux, F., Cerede, E., Gofflo, D., Gaillard-Kelly, M., and Philbert, D. (1994) *J. Steroid Biochem. Molec. Biol.* **48,** 111–119.
5. Neri, R. (1989) *Urology* **34 (suppl.),** 19–21.
6. Cockshott, I. D., Plummer, G. F., Cooper, K. J., and Warwick, M. J. (1991) *Xenobiotica* **21,** 1347–1355.
7. Mukherjee, A., Kirkovsky, L., Yao, X. T., Yates, C. R., Miller, D. D., and Dalton, J. T. (1996) *Xenobiotica* **26,** 117–122.
8. Tucker, H., and Chesterson, G. J. (1988) *J. Med. Chem.* **31,** 885–887.
9. Kirkovsky, L., Yin, D., Zhu, Z., Dalton, J. T., and Miller, D. D. (1998) *Amer. Chem. Soc. Natl. Mtg.,* Dallas, TX, March 29-April 2, in press.
10. Carlson, K. E., and Katzenellenbogen, J. A. (1990) *J. Steroid Biochem.* **36,** 549–561.
11. Wilson, E. M., and French, F. S. (1976) *J. Biol. Chem.* **251,** 5620–5629.
12. Mukherjee, A., Kirkovsky, L. I., Zhu, Z., Stourman, N., Kimura, Y., Miller, D. D., and Dalton, J. T. (1998) submitted.