Preclinical Pharmacology of a Nonsteroidal Ligand for Androgen Receptor-Mediated Imaging of Prostate Cancer

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ABSTRACT

Proper management of prostate cancer patients is highly dependent on the spread of the disease. High expression levels of the androgen receptor (AR) in prostate tumor offer a target for identifying cancer metastasis. We investigated the use of nonsteroidal AR ligands for receptor-mediated imaging as a diagnostic tool for prostate cancer staging. Compound S-26 [S-3-(4-fluorophenoxy)-2-hydroxy-2-methyl-N-(4-cyano-3-iodo-phenyl)-propionamide] was identified from a series of iodinated ether-linked derivatives of bicalutamide due to its high-AR binding affinity of 3.3 nM (which is similar to testosterone and ~25% of the binding affinity of dihydrotestosterone) in an in vitro competitive binding assay using rat prostate cytosol. Furthermore, S-26 exhibited a greater binding affinity ($K_i = 4.4$ nM) in a whole-cell binding assay using COS-7 cells transfected with human AR than testosterone ($K_i = 32.9$ nM) and dihydrotestosterone ($K_i = 45.4$ nM). We also confirmed that sex hormone-binding globulin (SHBG), a plasma protein that binds steroids with high affinity, does not bind with S-26. Cotransfection studies with the estrogen, progesterone, and glucocorticoid receptor indicated that S-26 does not cross-react with other members of the steroid hormone receptor family. The nonsteroidal structure, high-AR binding affinity, specificity, and lack of binding to SHBG indicate that S-26 exhibits favorable properties for further development as an imaging agent for prostate cancer.

Prostate cancer is the most common cancer and remains the second leading cause of death from cancer in American men (Greenlee et al., 2001). Procedures for reliable and accurate staging of prostate cancer are essential, because they greatly contribute to decisions regarding patient treatment and management. Because of the lack of adequate noninvasive diagnostic methods, many prostate cancer patients must undergo surgical staging to identify lymph node metastases (Kavoussi et al., 1993; Neal and Meis, 1994). The desire and search for noninvasive, more accurate, and selective imaging tools for prostate cancer provided the basis for these studies.

The androgen receptor (AR) is a member of the nuclear hormone receptor family, which includes the estrogen (ER), glucocorticoid (GR), mineralocorticoid, and progesterone (PR) receptors. Testosterone (T) and dihydrotestosterone (DHT), endogenous steroid ligands for the AR, regulate the growth and response of androgen-sensitive tissues. Upon androgen binding, the AR undergoes a conformational change, binds to specific DNA sequences, and modulates the transcription of target genes. Early immunohistochemical studies on human and rat tissues showed that the expression of AR is largely confined to the male reproductive organs, especially the prostate (Takeda et al., 1990). AR expression levels in benign prostatic hyperplasia and carcinoma samples vary widely (Shain et al., 1983; van Aubel et al., 1985; Bowman et al., 1986; Habib et al., 1986; Frydenberg et al., 1991). However, Brolin et al. (1992) observed a higher proportion of AR-positive cells in benign prostatic hyperplasia and prostate cancer metastases compared with normal tissues. Early work
on radiolabeled androgens demonstrated a selective uptake in the prostate of rats (Tveit and Attreamal, 1968; Symes, 1982; Carlson and Katzenellenbogen, 1990). Furthermore, preclinical studies using steroidal AR ligands in baboons also indicated that AR ligands bind the AR in vivo and were selectively retained in the prostate (Bonasera et al., 1996). The expression of AR in all stages of prostate cancer, regardless of tumor sensitivity to hormonal therapy (Sadi et al., 1991; van der Kwast et al., 1991; Van der Kwast et al., 1996) and the poor pharmacokinetics and specificity of steroidal ligands (Berger et al., 1975; Salman and Chammess, 1991; Choe et al., 1995; Labaree et al., 1999), are the basis of our approach to prostate cancer imaging. Nonsteroidal high-affinity AR ligands containing radioactive iodine provide a rational means for diagnostic imaging and potentially radiotherapy of prostate cancer. The relative lower energy (27 keV) and longer half-life of iodine-125 (60 days) offers a good radiotracer for imaging research, whereas iodine-123 (159 keV, with a half-life of 13 h) could be clinically used for diagnosis and iodine-131 (higher energy 364 keV, with a half-life of 8 days) could be used for receptor-mediated radiation therapy.

Our laboratories continue to explore the preclinical pharmacology of selective androgen receptor modulators or SARMs. We designed and synthesized many nonsteroidal compounds (Table 1) were synthesized and characterized in our laboratories as described previously (Nair et al., 2004, 2005). Reported here are the in vitro evaluation and characterization of one of the most promising compounds, S-26, as a potential imaging agent for prostate cancer.

**Materials and Methods**

**Chemicals.** Nonsteroidal compounds (Table 1) were synthesized and characterized in our laboratories as described previously (Nair et al., 2004, 2005). The purities of synthesized compounds were confirmed by NMR, elemental analysis, and mass spectrometry. 17a-methyl-[3H]Mibolerone ([3H]MIB, 84 Ci/mmol) and unlabeled MIB were purchased from PerkinElmer Life Sciences (Boston, MA). Hydroxypatite (HAP) was purchased from Bio-Rad Laboratories (Hercules, CA). EcoLite(+) scintillation cocktail was purchased from ICN Research Products Division (Costa Mesa, CA). Ethyl alcohol (United States Pharmacopeia grade) was purchased from AAPER Alcohol and Chemical Company (Shelbyville, KY). All other chemicals were purchased from Sigma (St. Louis, MO).

**Cell Culture.** Dulbecco’s modified Eagle’s medium (DMEM), RPMI 1640 medium, trypsin-EDTA, nonessential amino acids, and L-glutamine were purchased from Mediatech (Herndon, VA). Fetal bovine serum (FBS) and Lipofectamine reagent were purchased from Invitrogen (Carlsbad, CA). The monkey kidney fibroblast-like CV-1 (American Type Culture Collection, Manassas, VA). The cells were grown at 37°C in a humidified atmosphere with 5% carbon dioxide.

**AR Competitive Binding Assay.** The AR binding affinity of synthesized nonsteroidal compounds was determined using a radio-ligand competitive binding assay as reported previously (Yin et al., 2003b). In brief, an aliquot of AR cytosol preparation (50 μl) was incubated with a saturating concentration (1 nM) of [3H]MIB at 4°C for 18 h in the absence or presence of increasing concentrations of the compound of interest (10 different concentrations ranging from 10−2 to 104 nM). Triamcinolone acetonide (1000 nM) was included in the incubate to block the interaction of [3H]MIB with GR and PR. Non-specific binding of [3H]MIB was determined in separate incubates by adding an excess of unlabelled MIB (1000 nM) to the incubate. After incubation, the protein-bound radioactivity was separated from free radioactivity by HAP precipitation. The bound radioactivity was then extracted from HAP by incubating the HAP pellet with 1 ml of ethanol at room temperature for 1 h. The radioactivity was counted in a Beckman LS6800 liquid scintillation counter (Beckman Coulter, Fullerton, CA).

The specific binding of [3H]MIB at each concentration of the compound of interest (B) was obtained after subtracting the nonspecific binding of [3H]MIB and expressed as the percentage of the specific binding in the absence of the compound of interest (B0). The concentration of compound that reduced the specific binding of [3H]MIB (B0) by 50% ([IC50]) was determined by computer-fitting the data to the following equation using WinNonlin (Pharsight Corporation, Mountain View, CA): B = B0 × [1 − C/IC50 + C], where C was the concentration of the compound of interest. WinNonlin was provided by a Pharsight Academic License to The Ohio State University. The apparent equilibrium binding constant (Kd) of the compound of interest was calculated by Kd = IC50/([IC50]/Kd + L), where L was the equilibrium dissociation constant of [3H]MIB (Kd = 0.19 nM; determined in preliminary experiments) and L was the concentration of [3H]MIB used in the experiment (L = 1 nM).

**Whole-Cell Binding Assay.** COS-7 cells were transiently transfected with human AR (plasmid pCMV-hAR was generously provided by Dr. Donald Tindall, Mayo Clinic and Mayo Foundation, Rochester, MN). In brief, transfection was conducted in 150-mm diameter dishes using serum-free medium and Lipofectamine ac-
According to the manufacturer's instructions. The second day after transfection, COS-7 cells were seeded in 24-well plates and incubated for 1 day in a humidified CO₂ (5%) environment to reach greater than 80% confluence. The complete DMEM was then exchanged to serum-free and phenol red-free DMEM. One hour later, the cells were incubated with 1 nM [³H]MIB in the absence or presence of the ligand of interest (final concentration ranging from 10⁻¹ to 10⁶ nM). After a 4-h incubation at 37°C, the cells were washed three times with ice-cold PBS and lysed in 400 µl of 1 N NaOH. Radioactivity present in an aliquot (100 µl) of the lysate was then counted in a liquid scintillation counter. Cell number was normalized with the protein concentration in each well as determined by the BCA method. Nonspecific binding of [³H]MIB was determined in separate wells by adding an excess of unlabeled MIB (1000 nM) to the incubation medium. The specific binding of [³H]MIB at each concentration of the compound of interest was obtained after subtracting the nonspecific binding of [³H]MIB and expressed as the percentage of the specific binding in the absence of the compound of interest. The equilibrium dissociation constant Kᵣ of [³H]MIB in AR-transfected COS-7 was 0.5 nM, which was determined in preliminary experiments. Apparent Kᵣ values were calculated as described above in the AR competitive binding assay.

**Transactivation Studies with AR, ER, GR, and PR.** The in vitro functional activities of nonsteroidal ligands, as assessed by the ability of each ligand to induce or repress AR-mediated transcriptional activation of a hormone-dependent luciferase reporter gene, were examined in transiently transfected CV-1 cells. To determine the AR transactivation activities, CV-1 cells were maintained in DMEM containing 10% FBS, 0.1 mM nonessential amino acids, and 1% t-glutamine at 37°C in a humidified atmosphere containing 5% CO₂. Transient transfections of CV-1 cells were conducted in 150-mm diameter dishes using serum-free medium and Lipofectamine according to the manufacturer's instructions. Cotransfection was done by adding AR expression vector (plasmid pCMV-hAR was provided by Dr. Donald Tindall, Mayo Clinic and Mayo Foundation, Rochester, MN), luciferase reporter vector (pMMTV-Luc was provided by Dr. Ronald Evans, The Salk Institute, San Diego, CA), control β-galactosidase vector (pSV-β-galactosidase was obtained from Promega Corporation, Madison, WI), and Lipofectamine. At the time of transfection, medium was replaced with transfection medium (DMEM containing 1% t-glutamine) and the DNA/Lipofectamine solution. After 3 to 5 h of transfection, cells were washed once with DMEM and then recovered in fresh DMEM supplemented with 0.1% FBS for 10 to 12 h. After recovery, the transfected cells were distributed into 24-well plates at a density of 8 × 10⁴ cells per well. To start the transactivation studies, the medium was aspirated and replaced with 1 ml/well of medium containing the desired concentration of ligands. Negative control wells included no drug treatment, whereas positive control wells included 1 nM DHT only. After 24 h, the medium was aspirated, wells were washed twice with 1 ml/well PBS, and cells were lysed by incubation with 150 µl/well Reporter Lysis buffer (Promega) for 30 min at room temperature. An aliquot (50 µl) of the lysate in each well was sampled to measure luciferase and β-galactosidase activity.

For transactivation studies with ER, PR, and GR, the AR plasmid was replaced with expression vectors for ER, GR (p-hERₐ, p-hERₜ, and p-hGR were generously provided by Dr. Ronald Evans), and PR (p-hPR was generously provided by Dr. Donald P. McDonnell, Duke University, Durham, NC). Estradiol, progesterone, and dexamethasone were used as positive controls, respectively. Transcriptional activation in each well was calculated as the ratio of luciferase activity to β-galactosidase activity to normalize the variance in cell number and transfection efficiency. All experiments were performed in triplicate or greater, and data were expressed as the mean ± S.D. in representative experiments.

**AR Stability and Western Blot Analysis.** COS-7 cells were transiently transfected with AR in 150-mm diameter dishes and distributed in 12-well plates at a density of 2 × 10⁵ cells/well. The second day after transfection, the transfected COS-7 cells were incubated in serum-free phenol red-free DMEM containing DHT (10 nM), testosterone (10 nM), S-26 (1 or 10 nM), or drug-free medium for 10 h. Protein synthesis was blocked by coincubation with cycloheximide (50 µg/ml) at 37°C. After incubation, cells were washed with ice-cold PBS and lysed in 20 µM Tris-Cl, pH 7.4, 1% deoxycholate, 0.1% SDS, 150 µM NaCl, 1 mM EDTA, and 1% Triton X-100, supplemented with commercially available protease inhibitor cocktail (Sigma). Cell lysates were centrifuged, and the supernatant was used for protein measurement and Western blot analysis. The antibodies used were anti-AR polyclonal antibody N-20, actin polyclonal IgG (Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-rabbit IgG conjugated with horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

**SHBG Protein-Binding Studies.** SHBG binding studies were conducted using methods similar to those reported by Winneker et al. (1990). Human SHBG was prepared from human serum and precipitated by ammonium sulfate. Triplicate aliquots were incubated for 2 h in ice with [1,2-³H]DHT in either the absence or the presence of increasing concentrations (10⁻² to 10⁴ nM) of the compound of interest. At the end of incubation, bound radioactivity was separated by the HAP method and was extracted with ethanol. The radioactivity representing AR-bound [³H]DHT was counted in a Beckman LS6800 liquid scintillation counter (Beckman Coulter, Fullerton, CA). Nonspecific binding of [³H]DHT was determined by including an excess (10 µM) of unlabeled DHT in separate incubates.

**Results**

In Vitro AR Binding Affinity Determination. We designed and synthesized a series of iodinated nonsteroidal AR ligands (Table 1) based on the structure-activity relationship for nonsteroidal AR binding obtained from the literature and previous studies in our laboratories (Tucker et al., 1988; Dalton et al., 1998; Mukherjee et al., 1999; Kirkskovsky et al., 2000; Bohl et al., 2004). The AR binding affinities of these synthetic molecules, reported as apparent Kᵣ values, were determined by a radioligand competitive binding assay using rat prostates as an AR source (Table 1). Competitive binding studies showed that S-26 has high-AR binding affinity with an apparent Kᵣ of 3.3 ± 0.1 nM [i.e., a relative binding affinity of 24.9 ± 2.6% compared with DHT]. We previously reported that the presence of electron-withdrawing groups in the A ring is important for the binding affinity of nonsteroidal ligands, with compounds incorporating a CF₃ substituent at the R1 position of the A ring demonstrating high-binding affinity to the AR (Marhefka et al., 2004). The R2 position of B ring also plays an important role in AR binding affinity (Kim et al., 2005). When comparing compounds with identical substituents at the R2 position of the B ring, the binding affinity of compounds incorporating an iodine atom at position R1 of the A ring was very similar to that observed for compounds incorporating a trifluoromethyl substituent at this position (Table 1). One exception to this observation was the compound incorporating a chloro substituent at R2. In this case, the compound with a CF₃ group at R1 (i.e., S-31) bound the AR with much higher affinity (Table 1) than the iodine-containing compound (i.e., S-27).

**AR Whole-Cell Binding Affinity.** Because in vivo access to the AR will also be affected by the ability of the compounds to penetrate the cell membrane, we also examined the apparent binding affinity of the compounds in living cells. We performed whole-cell binding studies in COS-7 cells transfected with the human AR. To our surprise, the nonsteroidal
ligand S-26 demonstrated greater binding affinity and a much smaller $K_i$ value (4.3 ± 0.1 nM) than either DHT (45.4 ± 1.1 nM) or testosterone (32.9 ± 2.6 nM) in living cells (Fig. 1).

Transactivation Studies of AR, ER, GR, and PR. We next sought to determine whether S-26 is specific for the AR, as the other hormone receptors share significant homology with the ligand binding domain of the AR. For these studies, CV-1 monkey kidney cells (a cell line devoid of steroid hormone receptors) were transfected with expression plasmids for estrogen receptor $\alpha$ (ER$\alpha$), estrogen receptor $\beta$ (ER$\beta$), PR, or GR and their respective hormone-dependent luciferase reporter plasmids. Transfected cells were treated with the known agonist for each receptor (i.e., estradiol for ER$\alpha$ and ER$\beta$, progesterone for PR, and dexamethasone for GR) and increasing concentrations of S-26. Transfected cells were treated with the known agonist for each receptor (i.e., estradiol for ER$\alpha$ and ER$\beta$, progesterone for PR, and dexamethasone for GR) and increasing concentrations of S-26. Our preliminary data indicated that DHT elicits maximal AR-mediated transcriptional activation at a concentration of 1 nM and that higher concentrations were unable to stimulate further increases. We found that S-26 is an AR agonist, albeit with significantly lower potency than DHT. High (1000 nM) concentrations of S-26 were required to stimulate AR-mediated transcriptional activity to the same extent as 1 nM DHT. It is noteworthy that S-26 did not stimulate or inhibit agonist-induced transcriptional activation for ER, PR, and GR at biologically relevant concentrations (Fig. 2), although 1000 nM S-26 exhibited weak PR antagonist activity. Separate studies (Fig. 3) showed that S-26 significantly ($p < 0.01$) enhanced the AR-mediated transcriptional activation induced by 0.001 nM DHT and that the ability of S-26 to stimulate AR-mediated transcriptional activity was inhibited by bicalutamide (1 or 10 $\mu$M), providing further evidence of the specific interaction of S-26 with the AR. These data indicated that S-26 is specific for the AR and does not interact with other steroid hormone receptors.

Determination of Ligand-Dependent AR Stability. AR stability in the presence of DHT (10 nM), T (10 nM), and S-26 (10 nM) was examined in COS-7 cells transfected with the human AR. After 10 h of incubation at 37°C, the cells were lysed and protein expression was examined by Western blot assay. Figure 4 demonstrates that no decrease in the expression of AR level was observed in presence of either DHT, T, or S-26. This suggests that S-26 is a high-affinity ligand for the AR but does not cause destabilization of AR level in living cells, as previously observed for bicalutamide (Waller et al., 2000; Furutani et al., 2002).

SHBG Binding. We determined the binding affinity of S-26 and two steroidal androgens (testosterone and DHT) to SHBG, a plasma protein known to avidly bind steroidal androgens (Akvakumov et al., 2002). By comparison, testosterone bound to SHBG, with approximately 20% of the binding affinity of DHT. However, S-26 bound very poorly to SHBG, with less than 0.2% of the binding affinity of DHT (Fig. 5). These data suggest that rats are a valid animal model to predict S-26 biodistribution and pharmacokinetics in humans, as interaction of S-26 with SHBG is unimportant.

Discussion

Obtaining adequate information on the biological and physiological status of tumors is essential for the management and treatment of cancer patients. Targeting the AR with radioligands provides an approach potentially more sensitive than computerized tomography or magnetic resonance imaging for prostate cancer staging. The majority of steroid ligands were shown to be poor candidates for AR-mediated imaging because of their low-binding affinities, rapid metabolism, and lack of stability (Berger et al., 1975; Brandes and Katzenellenbogen, 1987; Salman and Chamness, 1991; Liu et al., 1992; Choe et al., 1995; Labaree et al., 1999). Androgenic steroids bind to other steroid receptors and SHBG in plasma, contributing to their poor target site specificity for imaging. Although SHBG has a high-binding affinity to most endogenous and synthesized androgens, it does not bind to the nonsteroidal androgen receptor ligands reported herein. In addition, reactivity with other steroid receptors is less prevalent with nonsteroidal compounds, providing another advantage over steroidal agents.

We developed a new series of ether-linked AR ligands, with improved metabolic stability and AR binding affinity as potential imaging agents. Previous literature reports demonstrated that a variety of substitution patterns can be used to modulate AR binding affinity (He et al., 2002; Bohl et al., 2004; Marhefka et al., 2004). For example, multiple substitutions on the B ring (Table 1) are possible with halogens, permitting the protection of this ring from oxidative metabolism (Kim et al., 2005). In addition, the para position of the A ring accommodates a variety of hydrogen bond acceptor groups, with nitro and cyano substitution resulting in compounds with greater binding affinity than halogens. It is noteworthy that the R1 position of the A ring may be modified to include a variety of electronegative substituents, including halogens. S-26 demonstrated that an iodine atom can be effectively incorporated on the A ring at the R1 position to maintain high-AR binding affinity (i.e., $K_i$ of 3.3 ± 0.1 nM, relative binding affinity = 24.9 ± 2.6% of that for DHT). The crystal structure of the trifluoromethyl (at the R1 position) analog of S-26 and the antagonist R-bicalutamide complexed to the AR demonstrate that the iodine would be accommodated in the same hydrophobic region occupied by the tri-
fluoromethyl group (Bohl et al., 2005a,b), explaining its favorable interaction with the AR. Surprisingly, S-26 demonstrates higher binding affinity than T and DHT in a whole-cell binding assay with human AR. The reasons for this finding are not likely due to the differences in rat and human AR, because the ligand binding domain is 100% identical in sequence (Sack et al., 2001). Further studies are needed to fully understand why DHT has a decreased apparent binding affinity compared with S-26 in whole-cell binding experiments. More importantly, S-26 maintains high-binding affinity in whole-cell binding studies, demonstrating its ability to efficiently target intracellular AR.

SHBG is a plasma glycoprotein found in many species that demonstrates high-binding affinity for certain estrogens and androgens. Although it is not expressed in rats, it is present at high concentrations in human serum (the ultimate milieu in which our imaging agents will be used). SHBG affects the pharmacokinetics of most steroidal ligands by sequestering them in the blood. We determined the binding affinity of S-26 and two steroidal androgens (i.e., testosterone and DHT) to
target tissue binding sites. In addition, we performed in vivo studies with a number of compounds, with similar chemical structures demonstrating their favorably pharmacokinetic and pharmacodynamic properties (Yin et al., 2003a; Kearbey et al., 2004; Gao et al., 2005) suggesting that S-26 will be efficacious in vivo.

We also examined the interaction of S-26 with other members of the nuclear hormone receptor family, because interaction with nontarget proteins would interfere with AR-mediated uptake in target tissues. S-26 did not stimulate or inhibit agonist-induced transcriptional activation for any of these nuclear hormone receptors at biologically relevant concentrations (S-26 slightly inhibited PR activation at a concentration of 1000 nM). These data indicate that S-26 is specific for the AR and will not interact with nontarget nuclear hormone receptors.

Stability is an important factor for a successful receptor-mediated imaging studies. We found that S-26 is very stable in mouse, rat, and human plasma at 37°C up to at least 48 h (data not shown). We also found that AR is very stable in the presence of DHT, T, or S-26. The well known LNCaP prostate cancer cell line contains a mutated AR. To examine the binding of our nonsteroidal ligands in this cell line, we performed whole-cell binding studies in LNCaP cells, and the results were similar to what we found in COS-7 cells transfected with wild-type AR (data not shown). In conclusion, the high affinity for the AR in a whole-cell binding assay, poor affinity to other steroid receptors and SHBG, and a stabilizing effect on the AR provide incentive for the development of S-26 as an AR-mediated imaging agent for prostate cancer.

References


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