Brief Articles

Design, Synthesis, and Pharmacological Characterization of 4-[4,4-Dimethyl-3-(4-hydroxybutyl)-5-oxo-2-thioxo-1-imidazolidinyl]-2-iodobenzonitrile as a High-Affinity Nonsteroidal Androgen Receptor Ligand[#]

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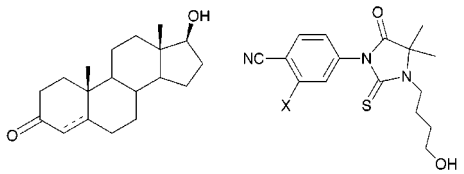
4-[4,4-Dimethyl-3-(4-hydroxybutyl)-5-oxo-2-thioxo-1-imidazolidinyl]-2-trifluoromethylbenzonitrile (RU 59063) is a prototype of a new class of high-affinity nonsteroidal androgen receptor (AR) ligands. The search for a radioiodinated AR ligand prompted us to synthesize 4-[4,4-dimethyl-3-(4-hydroxybutyl)-5-oxo-2-thioxo-1-imidazolidinyl]-2-iodobenzonitrile (DTIB) wherein the trifluoromethyl group of RU 59063 was substituted with the similarly hydrophobic iodine atom. DTIB displayed subnanomolar binding affinity ($K_i = 0.71 \pm 0.22$ nM) for the rat AR in competitive binding assays. Additionally, DTIB demonstrated potent agonist activity, comparable to that of the natural androgen 5α-dihydrotestosterone (DHT), in a cell-based functional assay (cotransfection assay). DTIB represents a new lead for the development of high-affinity radioiodinated AR radioligands.

Introduction

The androgen receptor (AR) is an important member of the superfamily of nuclear hormone receptors that function as ligand-dependent regulators of transcription.[1] ARs respond to signaling by the endogenous steroidal androgens testosterone and 5α-dihydrotestosterone (DHT) and play a critical role in sexual development and function in males.[2]

The development and progression of prostate cancer is known to be androgen-dependent, and AR expression is frequently observed in primary prostate tumors and metastases.[3] Consequently, AR-targeted radioligands are under investigation for the noninvasive imaging of tumor sites in prostate cancer using positron emission tomography (PET) and single-photon emission computed tomography (SPECT).[4] The majority of these studies to date have focused on steroid-based ligands, including the naturally occurring steroid ligands (testosterone, DHT) and synthetic steroids [mibolerone, metribolone (R 1881)].[5] Successful PET imaging of prostate and prostate tumor metastases has been recently reported in a preliminary clinical study with a fluorine-18-labeled derivative of DHT.[6] In contrast, the development of high-affinity radioiodinated steroid-based AR ligands for SPECT imaging has had limited success. This is attributed, in part, to the sensitivity of the AR ligand binding domain to the bulky nature of iodinated steroids.[7]

Chart 1. Structures of AR Ligands



Δ[4,5]; Testosterone (**T**) — X = CF_3; RU 59063

Dihydrotestosterone (**DHT**) — X = I; DTIB (**1**)

Nonsteroidal antiandrogens offer an alternative approach to the design of high-affinity radioiodinated AR ligands. Since nonsteroidal AR ligands have greater conformational flexibility and are more amenable to structural modification than steroid-based ligands, they could serve as templates for the design of iodinated ligands that are better accommodated at the AR ligand binding domain. RU 59063 (Chart 1) is a prototype member of a novel class of nonsteroidal antiandrogens which displays high AR affinity ($K_a = 5.4$ nM for human AR) and selectivity (>1000-fold selectivity for AR over progestin, glucocorticoid, mineralocorticoid, and estrogen receptors).[8] In addition, [^{3}H]RU 59063 demonstrated high specific binding and 3−8-fold greater AR binding affinity as compared to [^{3}H]testosterone in radioligand binding assays conducted with rat, mouse, hamster, and human AR tissue preparations.[8]

The high AR selectivity, reduced lipophilicity, and ease of synthesis of RU 59063 and its derivatives as compared to steroid analogues make these compounds attractive candidates for investigation as radioiodinated AR radioligands. As a first step toward this goal, we

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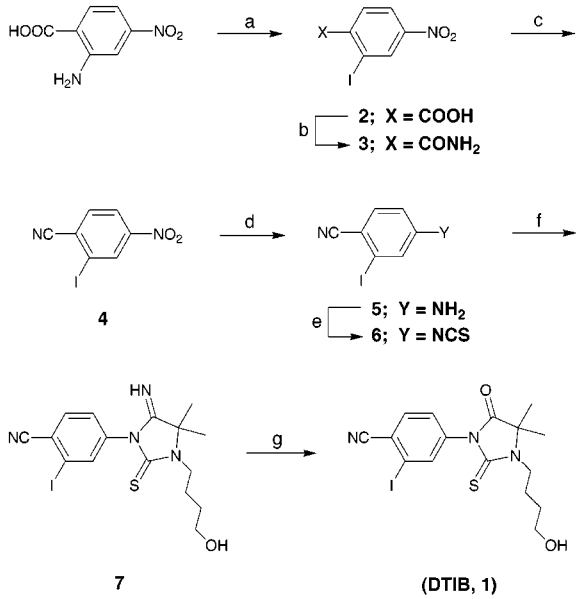
Scheme 1[a]



[a] Reagents and conditions: (a) 1. $NaNO_2$, H_2SO_4, 0 °C, 2. NaI; (b) 1. $SOCl_2$, 60 °C, 2. NH_4OH; (c) $SOCl_2$, reflux; (d) $SnCl_2$, EtOH, reflux; (e) $CSCl_2$, THF:H_2O, rt; (f) 2-(4-hydroxybutyl)amino-2-cyanopropane, Et_3N, THF, reflux; (g) 2 N HCl, CH_3OH, reflux.

selected the iodinated derivative 4-[4,4-dimethyl-3-(4-hydroxybutyl)-5-oxo-2-thioxo-1-imidazolidinyl]-2-iodobenzonitrile (DTIB) (Chart 1) for initial evaluation based on the similar hydrophobic properties of the trifluoromethyl ($\pi = 0.88$) and iodine ($\pi = 1.12$) groups.[9] We report here the synthesis of DTIB and its initial in vitro pharmacological characterization (AR binding, AR-mediated transcriptional activation) as a novel high-affinity, nonsteroidal AR ligand.

Chemistry

DTIB (**1**) was prepared by a seven-step synthetic route as outlined in Scheme 1. Commercially available 2-amino-4-nitrobenzoic acid was converted to 2-iodo-4-nitrobenzoic (**2**) in moderate yield (42%) via the Sandmeyer diazotization reaction. Sequential reaction of **2** with thionyl chloride and ammonia provided 2-iodo-4-nitrobenzamide (**3**). Dehydration of **3** was accomplished with thionyl chloride to give the nitrile **4,** which was subsequently reduced to the aniline derivative **5** with stannous chloride in EtOH. Synthesis of the isothiocyanate derivative **6** and subsequent steps were conducted according to the general procedure of Teutsch et al.[8] Thus, treatment of **5** with thiophosgene gave the isothiocyanate **6** which was condensed with 2-(4-hydroxybutylamino)-2-cyanopropane in the presence of triethylamine to afford the imine intermediate **7**. Acid hydrolysis of **7** provided DTIB (**1**) as a white amorphous solid in 16**%** overall yield. DTIB and intermediate compounds were purified by silica gel flash chromatography and fully characterized by ^{1}H NMR, mass spectral, and elemental analysis.

Results and Discussion

The binding affinity of DTIB and RU 59063 to the rat prostate cytosolic AR was determined using a competitive binding assay in the presence of the high-affinity AR radioligand, [^{3}H]mibolerone.[10] These data and the binding affinity values obtained for other routinely used AR ligands such as DHT, mibolerone, and testosterone are presented in Table 1. As seen from these data, DTIB ($K_i = 0.71 \pm 0.22$ nM) is one of the highest-affinity AR ligands reported to date. DTIB inhibited [^{3}H]mibolerone binding to the rat AR as efficiently as mibolerone and DHT, two tritiated AR ligands currently in use. Furthermore, DTIB showed a 3-fold improvement in AR affinity over RU 59063 indicating that iodine substitution was well-tolerated in this region of the molecule. Hill coefficients (data not shown) were close to unity in all cases indicating that the binding interaction was to a single site.

Table 1. Inhibition Constants for DTIB (**1**) and Selected Ligands at the Rat AR[a]

compd	$K_i \pm$ SEM (nM)[b]
DTIB (**1**)	0.71 ± 0.22
RU 59063	2.23 ± 0.50
DHT	0.69 ± 0.20
testosterone	1.4 ± 0.4
mibolerone	0.75 ± 0.08

[a] Binding studies were conducted using rat prostate cytosol AR and [^{3}H]mibolerone as radioligand. [b] Data are presented as mean ± SEM and are the average of at least three determinations each conducted in duplicate.

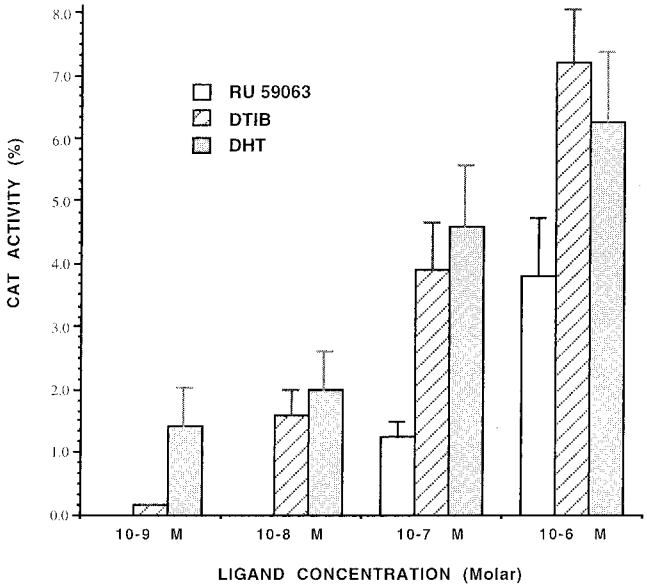


Figure 1. Comparison of AR agonist dose response of RU 59063, DTIB, and DHT in cotransfected CV-1 cells. Data points represent the mean ± SEM of triplicate determinations; chloramphenicol acetyltransferase (CAT) activity is reported as % conversion/μg of protein; RU 59063 was tested at two dose levels: 10^{-7} and 10^{-6} M.

The AR functional activity of DTIB, RU 59063, and DHT was evaluated using an in vitro cell-based assay (cotransfection assay) system.[11] DTIB and RU 59063 displayed a dose-dependent stimulation of AR-mediated transcriptional activity in these assays with a potency comparable to that of the natural hormone agonist DHT (Figure 1). Similar agonistic effects (data not shown) were observed for these ligands using a more complex natural gene fragment (C′Δ9, a transcriptional enhancer of the mouse sex-limited protein gene[12]). The agonistic behavior of RU 59063 seen in transfection experiments is in contrast to the potent in vivo AR antagonist activity reported for this analogue in rodent models.[8] A similar discrepancy between in vitro and in vivo functional

activity has been observed for the structurally similar antiandrogen RU 56187.[13]

Although our initial design strategy of DTIB was based on the similar hydrophobicity of iodine and trifluoromethyl, it must be noted that these functional groups do not share similar electronic and steric properties. In particular, iodine is less electronegative (σm, σp values are 0.35 and 0.18, respectively, for iodine versus 0.43 and 0.54, respectively, for CF_3) and has a 3-fold larger steric size than trifluoromethyl (molar refractivity values for iodine and CF_3 are 13.9 and 5.0, respectively).[9] Our binding affinity data therefore indicate that the AR binding domain that interacts with the trifluoromethyl substituent in RU 59063 is sufficiently large to accommodate the increased steric bulk of an iodine atom. Taken together, these results suggest that steric and hydrophobic interactions may be more important than electronic interactions at this binding region for high AR binding in this class of nonsteroidal ligands.

Conclusions

In summary, the present study has identified a new iodinated nonsteroidal AR ligand, DTIB, which demonstrates subnanomolar binding affinity and potent agonist activity in in vitro AR binding and functional assays, respectively. These studies also suggest the existence of a large hydrophobic pocket in the AR binding domain that interacts with the *meta* substituent of this pharmacophore. Studies are currently underway to evaluate the utility of radioiodinated analogues of DTIB as radioligands for in vitro and in vivo studies of AR.

Experimental Section

General Methods. Melting points were determined with a Thomas-Hoover melting point apparatus and are uncorrected. ^{1}H NMR spectra were obtained in either $CDCl_3$ or CD_3OD with a Bruker WM-360 (360 MHz) instrument using tetramethylsilane (TMS) as internal standard. Chemical shifts (δ) are reported in parts per million (ppm) relative to TMS, and coupling constants (J) are reported in hertz (Hz). Elemental analyses were performed by the Department of Chemistry, University of Michigan, and were within ±0.4% of the calculated values. RU 59063 and 2-(4-hydroxybutylamino)-2-cyanopropane were synthesized as previously described.[8] All other chemical reagents were obtained from Aldrich Chemical Co., Milwaukee, WI, and were used without further purification. Organic extracts were dried over anhydrous Na_2SO_4 and concentrated to dryness by rotoevaporation under reduced pressure.

2-Iodo-4-nitrobenzoic Acid (2). A vigorously stirred slurry of 2-amino-4-nitrobenzoic acid (10 g, 55 mmol) and aqueous 9 N H_2SO_4 (64 mL) was diazotized at 0 °C (ice−salt bath) by dropwise addition of a solution of $NaNO_2$ (4.17 g, 60.4 mmol) in water (50 mL). The mixture was stirred at 0 °C for 1 h and treated with urea (1.1 g, 18.3 mmol) to destroy excess HNO_2. A solution of NaI (9.9 g, 66 mmol) in H_2O (50 mL) was then added dropwise at 0−5 °C. The reaction was allowed to warm to ambient temperature, stirred a further 2 h, diluted with 1% aqueous $NaHSO_3$ solution (200 mL) and filtered. The crude product was rinsed with hot EtOAc (3 × 100 mL) to remove a dark orange side product and the residue was purified by flash chromatography [gradient elution with EtOAc:hexane:glacial acetic acid (30:70:1 to 80:20:1)] to give 6.8 g (42%) of **2** as light yellow needles: mp 143.5−145 °C [benzene:hexane (1:1)]; ^{1}H NMR (CD_3OD) δ 8.77 (d, 1H, J = 2.2, H-3), 8.30 (dd, 1H, J = 8.5, 2.2, H-5), 7.92 (d, 1H, J = 8.5, H-6). Anal. ($C_7H_4NO_4I$) C, H, N.

2-Iodo-4-nitrobenzamide (3). A mixture of **2** (6.60 g, 22.5 mmol) and $SOCl_2$ (100 mL) was heated at 60 °C for 3 h under argon and concentrated by rotoevaporation. Residual $SOCl_2$ was removed from the crude product mixture by coevaporation with dry $CHCl_3$ (3 × 50 mL). Concentrated aqueous NH_3 (100 mL) was then added and the mixture stirred overnight at ambient temperature. The precipitate was filtered, rinsed with H_2O and dried in an oven at 60 °C. The crude product was purified by flash chromatography (75% EtOAc in hexane) to give 5.6 g (85%) of **3** as cream-colored crystals: mp 208−209 °C (EtOAc); ^{1}H NMR (CD_3OD) δ 8.70 (d, 1H, J = 2.2, H-3), 8.29 (dd, 1H, J = 8.5, 2.2, H-5), 7.59 (d, 1H, J = 8.4, H-6). Anal. ($C_7H_5N_2O_3I$) C, H, N.

2-Iodo-4-nitrobenzonitrile (4). A mixture of **3** (5.5 g, 18.8 mmol) and $SOCl_2$ (35 mL) was refluxed for 3 h under argon and concentrated under reduced pressure. The residue was purified by flash chromatography (20% EtOAc in hexane) to give 4.12 g (80%) of **4** as cream-colored crystals: mp 154−155.5 °C [EtOAc:hexane (1:4)]; ^{1}H NMR ($CDCl_3$) δ 8.76 (d, 1H, J = 2.2, H-3), 8.32 (dd, 1H, J = 8.5, 2.2, H-5), 7.82 (d, 1H, J = 8.6, H-6). Anal. ($C_7H_3N_2O_2I$) C, H, N.

4-Cyano-3-iodoaniline (5). A mixture of **4** (2.3 g, 8.4 mmol) and $SnCl_2 \cdot 2H_2O$ (9.3 g, 41.4 mmol) in EtOH (35 mL) was refluxed for 2 h. The mixture was concentrated under reduced pressure and treated with H_2O (100 mL) and the pH was adjusted to 9 by treatment with 5% aqueous $NaHCO_3$. The mixture was extracted with EtOAc; the organic layers were washed with saturated brine and H_2O and dried. The residue obtained after removal of solvent was purified by flash chromatography (40% EtOAc in hexane) to afford 2 g (98%) of **5** as white fluffy crystals: mp 146−148 °C [EtOH:H_2O (1:5)]; ^{1}H NMR ($CDCl_3$) δ 7.33 (d, 1H, J = 8.5, H-5), 7.14 (d, 1H, J = 2.3, H-2), 6.62 (dd, 1H, J = 8.5, 2.2, H-6), 4.19 (br s, 2H, exchangeable with D_2O, NH_2). Anal. ($C_7H_5N_2I$) C, H, N.

2-Iodo-4-isothiocyanatobenzonitrile (6). A solution of **5** (0.54 g, 2.2 mmol) in THF (10 mL) was added dropwise at ambient temperature to a well-stirred suspension of thiophosgene (0.30 g, 2.6 mmol) in H_2O (5 mL). TLC analysis [silica; hexane:EtOAc (6:1)] at 3 h indicated completeness of reaction. The mixture was diluted with H_2O (20 mL), extracted with $CHCl_3$ (2 × 20 mL) and dried. The residue obtained after removal of solvent was dried under high vacuum to afford a quantitative yield of a yellow-brown solid which was used directly in the next step. A small portion of the crude product was purified by flash chromatography (15% EtOAc in hexane) to give an analytical sample of **6** as a white solid: mp 116−118 °C; ^{1}H NMR ($CDCl_3$) δ 7.76 (d, 1H, J = 2.1, H-3), 7.58 (d, 1H, J = 8.3, H-6), 7.26 (dd, 1H, J = 8.3, 2.1, H-5); HRMS (EI) calcd for $C_8H_3N_2IS$ (M^+) 285.9062, found 285.9058.

4-[4,4-Dimethyl-3-(4-hydroxybutyl)-5-imino-2-thioxo-1-imidazolidinyl]-2-iodobenzonitrile (7). A solution of 2-(4-hydroxybutylamino)-2-cyanopropane[8] (0.30 g, 1.94 mmol) in anhydrous THF (2 mL) was added dropwise at ambient temperature to a stirred solution of the crude isothiocyanate **6** (0.55 g, 1.94 mmol) and Et_3N (25 mg, 0.25 mmol) in anhydrous THF (2 mL). The reaction mixture was refluxed for 1 h, at which point, TLC analysis [silica; hexanes:EtOAC (6:1)] indicated completeness of reaction. The reaction mixture was concentrated under reduced pressure and the residue purified by flash chromatography (35% acetone in chloroform) to provide 0.55 g (64%) of the title compound **7** as a straw-colored viscous liquid: ^{1}H NMR ($CDCl_3$) δ 7.93 (s, 1H, Ar−H), 7.75 (d, 1H, J = 8.3, Ar−H), 7.49 (d, 1H, J = 8.2, Ar−H), 3.75−3.66 (m, 4H, $-NCH_2-$ and $-CH_2O-$), 1.92 (m, 2H, CH_2), 1.66 (m, 2H, CH_2), 1.58 (s, 6H, $C(CH_3)_2$); HRMS (EI) calcd for $C_{16}H_{19}N_4ISO$ (M^+) 442.0324, found 442.0320.

4-[4,4-Dimethyl-3-(4-hydroxybutyl)-5-oxo-2-thioxo-1-imidazolidinyl]-2-iodobenzonitrile (DTIB, 1). A solution of the imine derivative **7** (0.48 g, 1.1 mmol) in CH_3OH (8 mL) was treated with aqueous 2 N HCl (1 mL) and refluxed for 1 h. The cooled reaction mixture was poured into H_2O (80 mL) and extracted with EtOAc (2 × 50 mL), and the organic layers dried and concentrated under reduced pressure. The crude product was purified by flash chromatography (70% EtOAc in

hexane) to give 0.44 g (90%) of DTIB (**1**) as a white amorphous solid: ^{1}H NMR ($CDCl_3$) δ 7.96 (d, 1H, J = 1.9, Ar−H3), 7.71 (d, 1H, J = 8.3, Ar−H6), 7.52 (dd, 1H, J = 8.3, 1.9, Ar−H5), 3.73 (m, 4H, $-NCH_2-$ and $-CH_2O-$), 1.94 (m, 2H, CH_2), 1.66 (m, 2H, CH_2), 1.57 (s, 6H, $C(CH_3)_2$). Anal. ($C_{16}H_{18}N_3O_2IS$) C, H, N.

Androgen Receptor Binding Assays. Androgen receptor binding assays were conducted by a commercial laboratory (MDS Panlabs, Bothell, WA) using [^{3}H]mibolerone as radioligand and rat prostate cytosol as a source of AR as previously described.[10] In brief, cytosol from the ventral prostate of castrated male Wistar rats (175 ± 25 g) was prepared in modified phosphate buffer (pH 7.2) containing protease inhibitors and triamcinolone acetonide (5 mM). Competitive binding of test ligands were determined by incubation of increasing concentrations (0.3−100 nM) of each ligand and a saturating concentration of [^{3}H]mibolerone (2 nM) with cytosol (0.4 mg) at 4 °C for 18 h. Separation of bound and free radioligand was achieved by further incubation with a hydroxylapatite slurry for 15 min and filtration. Radioactivity bound to the washed filters was quantified by liquid scintillation counting. Binding affinity data are reported as mean ± standard error of the mean (SEM) and represent the average of at least three separate experiments each conducted in duplicate.

Cotransfection Assays. AR-mediated transcriptional activation by ligands was studied in a cell-based bioassay system (cotransfection assays) as previously reported.[11] In brief, mammalian CV-1 cells were transfected with the mouse AR and an androgen-responsive reporter gene, consisting of three tandem androgen response elements upstream of the thymidine kinase promoter and the bacterial chloramphenicol acetyltransferase (CAT) gene. Media containing the reference compound (DHT) or test compound in concentrations ranging from 1 nM to 1 μM were added to the cells. Three independent transfection assays were performed. Following incubation, cells were washed with phosphate buffered saline and lysed by several cycles of freeze−thaw. Cell extracts were assayed for CAT activity and the data reported as CAT conversion rates (%).[11]

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