New Multi-target Antagonists of $\alpha_{1A}$-, $\alpha_{1D}$-Adrenoceptors and 5-HT$1A$ Receptors Reduce Human Hyperplastic Prostate Cell Growth and the Increase of Intraurethral Pressure$^\text{S}$


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ABSTRACT

Benign prostatic hyperplasia (BPH) is characterized by stromal cell proliferation and contraction of the periurethral smooth muscle, causing lower urinary tract symptoms. Current BPH treatment, based on monotherapy with $\alpha_{1A}$-adrenoceptor antagonists, is helpful for many patients, but insufficient for others, and recent reports suggest that stimulation of $\alpha_{1D}$-adrenoceptors and 5-hydroxytryptamine (serotonin) (5-HT)$1A$ receptors contributes to cell proliferation. In this study, we investigated the potential of three N-phenylpiperazine derivatives (LDT3, LDT5, and LDT8) as multi-target antagonists of BPH-associated receptors. The affinity and efficacy of LDTs were estimated in isometric contraction and competition-binding assays using tissues (prostate and aorta) and brain membrane samples enriched in specific on- or off-target receptors. LDTs' potency was estimated in intracellular Ca$^{2+}$ elevation assays using cells overexpressing human $\alpha_{1}$-adrenoceptor subtypes. The antiproliferative effect of LDTs on prostate cells from BPH patients was evaluated by viable cell counting and 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide assays. We also determined LDTs' effects on rat intraurethral and arterial pressure. LDT3 and LDT5 are potent antagonists of $\alpha_{1A}$-, $\alpha_{1D}$- adrenoceptors, and 5-HT$1A$ receptors (K$_i$ values in the nanomolar range), and fully inhibited phenylephrine- and 5-HT–induced proliferation of BPH cells. In vivo, LDT3 and LDT5 fully blocked the increase of intraurethral pressure (IUP) induced by phenylephrine at doses (ED$_50$ of 0.15 and 0.09 mg kg$^{-1}$, respectively) without effect on basal mean blood pressure. LDT3 and LDT5 are multi-target antagonists of key receptors in BPH, and are capable of triggering both prostate muscle relaxation and human hyperplastic prostate cell growth inhibition in vitro. Thus, LDT3 and LDT5 represent potential new lead compounds for BPH treatment.

Introduction

Benign prostatic hyperplasia (BPH) is a progressive disease with considerable impact on the quality of life of a large portion of aging men (Nickel, 2003). The condition stems from an imbalance between cell proliferation and apoptosis (Roehrborn, 2008; Sciarra et al., 2008). Among several factors, the proliferation of periurethral prostate stromal cells and the prostatic smooth muscle contraction contribute to the lower urinary tract symptoms (LUTS) suggestive of BPH (LUTS/BPH) (Roehrborn, 2008).

$\alpha_{1A}$-Adrenoceptor is the predominant subtype expressed in human prostate, particularly in the stroma (Price et al., 1993; Tseng-Crank et al., 1995), and mediates prostate muscle contraction (Forray et al., 1994). These receptors are also highly expressed in hyperplastic prostate, and, under this condition, their mRNA level corresponds to approximately 85% of the total prostate $\alpha_{1}$-adrenoceptor mRNA content (Nasu et al., 1996).

Both the American and European Urological Associations consider $\alpha_{1}$-adrenoceptor antagonism as an appropriate pharmacological treatment to control moderate to severe

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ABREVIATIONS: ANOVA, analysis of variance; BMY7378, 8-[2-[(4-[(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride; BPH, benign prostatic hyperplasia; [Ca$^{2+}$]$^{-1}$, intracellular Ca$^{2+}$ concentration; 8-OH-DPAT, 8-hydroxy-2-(dipropylamino)tetralin hydrobromide; 5-HT, 5-hydroxytryptamine (serotonin); IUP, intraurethral pressure; LUTS, lower urinary tract symptoms; MTT, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide; p-MPPF, 4-fluoro-N-[2-[(4-[(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-[2-pyridyl]benzamide dihydrochloride; RX821002, 2-methoxy idazoxan.
LUTS/BPH (McVary et al., 2011; Oelke et al., 2013). Nevertheless, the introduction of α1A-adrenoceptor antagonists such as tamsulosin, or the uroselective α1-adrenoceptor blocker alfuzosin, was a major advance in the management of BPH, mainly due to the better tolerability by patients (Michel 2010; Jelski and Speakman, 2012). However, the pharmacological management of LUTS/BPH by monotherapy with α1A-adrenoceptor antagonists is helpful for many patients, but insufficient for others (Jelski and Speakman, 2012; Perabo, 2012). Two important factors of LUTS/BPH not addressed by α1A-adrenoceptor blockers are sheen prostate size and detrusor muscle contraction (McVary et al., 2011; Oelke et al., 2013).

The possibility that α1D-adrenoceptors also might play a role in the pathogenesis of BPH has been suggested. According to data obtained by RNase protection, in situ hybridization or reverse-transcription polymerase chain reaction assays, the expression level of α1A-, α1B-, and α1D-adrenoceptor mRNA differs among hyperplastic prostate samples; however, the prostatic expression of α1D-adrenoceptor mRNA is frequently increased in such condition (Nasu et al., 1996; Kojima et al., 2006, 2009a). Some reports have suggested that α1D-adrenoceptor blockade may improve BPH treatment by inhibiting prostate cell growth in vitro and in vivo (Kojima et al., 2009a).

In contrast, tamsulosin, one of the most widely used drugs for BPH treatment, is not as effective in this model (Kojima et al., 2009). Some reports have suggested that α1D-adrenoceptor antagonists are helpful for many patients with LUTS/BPH and BPH during transurethral resection, in accordance with the Declaration of Helsinki (de Souza et al., 2011). Informed consent was obtained from donors (Ethics Committee of Universidade Federal do Rio de Janeiro, CAAE-0029.0.197.000-05, 2009). The androgen-independent prostate cancer line DU-145 (human) was obtained from the Rio de Janeiro Cell Bank (Universidade Federal do Rio de Janeiro).

Materials and Methods

Patient Samples and Human Cell Lines. Prostate tissue samples were collected from three patients with LUTS secondary to BPH during transurethral resection, in accordance with the Declaration of Helsinki (de Souza et al., 2011). Informed consent was obtained from donors (Ethics Committee of Universidade Federal do Rio de Janeiro, CAAE-0029.0.197.000-05, 2009). The androgen-independent prostate cancer cell line DU-145 (human) was obtained from the Rio de Janeiro Cell Bank (Universidade Federal do Rio de Janeiro).

Animals. All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals and with institutional ethical standards established by the Ethics Committee of the Federal University of Rio de Janeiro, under the license DPBC-ICB-011 (2008). Animals were kept under a 12/12-hour light/dark cycle, with water and food ad libitum, and in agreement with the guidelines of the National Council on Experimental Animal Control (CONCEA, Brazil) and the Committee of Care and Use of Laboratory Animals (National Research Council). Male Wistar rats (250–300 g; 2–3 months) were used in this study. For brain and liver removal, rats were anesthetized with ether and killed by decapitation.

Drugs and Radioligands. Prazosin hydrochloride, pargyline hydrochloride, 5-hydroxytryptamine hydrochloride (5-HT), acetylcholine chloride, (R)-(-)-phenylephrine hydrochloride, L-adrenaline (Hampel et al., 2002; Barendrecht et al., 2009).

In the case of multifactorial diseases such as BPH (Roehrborn, 2008), a multi-target strategy seems more appropriate (Peters, 2013). For the treatment of BPH, the use of antagonists that concomitantly relax the prostate and slow prostate enlargement might be more effective than monotherapy targeting solely the α1A-adrenoceptors (Hieble, 2011), so that we hypothesized that not only α1D-adrenoceptors, but also 5-HT1A receptors, could be additional targets. In fact, neuroendocrine cells populate normal and malignant prostate tissue releasing 5-HT (Abrahamsson et al., 1986), and prostate cells, including those from BPH patients, express 5-HT1A receptors (Dizeyi et al., 2004). Moreover, 5-HT1A receptor activation appears to increase prostate cell proliferation, via stimulation of the Akt/mitogen-activated protein kinase pathway (Hsiung et al., 2005; Dizeyi et al., 2011), and the 5-HT1A receptor antagonist NAN190 reduces prostate cell proliferation (Dizeyi et al., 2004). Based on these data, 5-HT1A receptors are considered as an attractive target for drug development in such context (Fiorino et al., 2014).

Previously, we showed that the N1-(2-methoxyphenyl)-N4-piperazine moiety confers affinity for α1A-, α1D-adrenoceptors, and 5-HT1A Receptors (Chagas-Silva et al., 2014). In this work, we investigate the in vitro and in vivo pharmacological characteristics of three N-phenylpiperazine derivatives, LDT3, LDT5, and LDT8 (European patent office, application 13733873.7-1451; USPTO application 14370646). Our results show that LDT3 and LDT5 are very potent multi-target antagonists of both α1A- and α1D-adrenoceptors, and also of 5-HT1A receptors. Also, these compounds inhibit the increase of rat IUP (as a result of prostate contraction) in vivo and human hyperplastic prostate cell proliferation in vitro. As a conclusion, we elected the multi-target LDT3 and LDT5 as potential lead compounds to reduce LUTS/BPH and BPH progression.
and denuded aorta segments were preloaded (60 minutes) with 10 or 20 mM, respectively, and washed twice. Tissues were contracted with 1 μM phenylephrine (aorta) or 60 mM KCl depolarizing solution (prostate). After a 60-minute recovery period, aorta and prostate samples were contracted with cumulative concentrations of phenylephrine (10^-9–10^-3 M) in the presence of 1 μM propranolol, before and after incubation for 60 minutes with the test compounds (10 or 50 nM), BMY7378 or tamsulosin (10 nM). The developed force was recorded using a FT-03 grass force transducer (Warwick, RI) connected to a data acquisition system (PowerLab, ADInstruments, Bella Vista, Australia). Data were analyzed by nonlinear regression (GraphPad Prism 5.0; GraphPad Software, San Diego, CA), and the maximal contraction obtained in each control curve (before treatment) was defined as the top. The phenylephrine concentration that produced 50% of the maximal contraction (EC50) was estimated before and after treatment with the antagonists. An EC50 ratio (CR) was calculated for the control value (before treatment). Drug affinity (Kd) was estimated using the Schild equation: log \( \frac{C_{R}-1}{C_{R}} \) = log \[ B \] - log \( K_d \) (Kenakin, 1993), where B is the antagonist.

**Intracellular Ca\(^{2+}\) Measurement.** The effect of LDTs on human \( \alpha_1 \)-adrenoceptor subtypes was determined by measuring intracellular Ca\(^{2+}\) in rat-1 fibroblasts stably expressing \( \alpha_1A, \alpha_1B, \) or \( \alpha_1D \)-adrenoceptors (Vázquez-Prado et al., 1997). These cells were cultured in high-glucose modified Eagle’s medium with L-glutamine supplemented with 10% fetal bovine serum, 300 μg/ml neomycin analog G418 sulfate, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.25 μg/ml amphotericin B, at 37°C, and under a 5% CO\(_2\) atmosphere.

Cells were loaded with 2.5 μM fura-2/AM for 60 minutes at 37°C, in 20 mM HEPES (pH 7.4) containing 120 mM NaCl, 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 4.75 mM KCl, 10 mM glucose, 1.2 mM CaCl\(_2\), and 0.05% bovine serum albumin (pH 7.4). Then cells were detached by gentle trypsinization, washed to remove unincorporated dye, and incubated (10^6 cells / condition) with vehicle, LDTs (10^-9–10^-5 M), BMY7378 (10^-9–10^-6 M), or tamsulosin (10^-10–10^-7 M) for 100 seconds, before stimulation with 100 μM phenylephrine. Fluorophore excitation was performed at 340 and 380 nm and measured at 510 nm emission wavelength, at 0.5-second intervals, using an Aminco-Bowman Series 2 luminescence spectrometer (Rochester, NY). Peak fluorescence values were used for data analysis, and the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) was calculated, as described previously (Gryniewicz et al., 1985). Data were analyzed by computerized nonlinear regression of untransformed data (GraphPad Prism 5.0; GraphPad Software), to estimate the IC50 of test compounds based on individual curves obtained from n experiments.

**Intraurethral and Blood Pressure Assays.** Male Wistar rats were cannulated, as described previously (Chagas-Silva et al., 2014). For these assays, each animal was used only once, and all drugs were diluted in isotonic saline. Blood and IUP (in mmHg) were monitored continuously using a fluid-filled pressure transducer (PowerLab, ADInstruments, Bella Vista, Australia).

Rats were anesthetized with sodium pentobarbital (60 mg kg\(^{-1}\) body weight, i.p.), and body temperature was kept constant at 37°C. The jugular vein was cannulated with polyethylene cannulae containing a heparinized saline solution (50 U/ml). Anesthesia was complemented with sodium pentobarbital before treatments. The pressure transducer was placed into the right carotid artery. After blood pressure stabilization (30 minutes), 0.1 μg kg^-1 LDT3, 0.1–100 μg kg^-1 LDT5, or 100 μl vehicle (saline) was injected i.v. in bolus. Data were analyzed by LabChart software. Mean arterial (blood) pressure was calculated by arithmetic mean of the diastolic and systolic pressures in the respective cycles, and changes were expressed as percentage of the alteration of the baseline (resting) values (130.2 ± 2.2 mmHg, n = 20).

For IUP determination, the prostate and bladder were exposed through a midline incision in the lower abdomen. The pressure catheter was placed into the prostatic urethra through the bladder and fixed at the vesical-urethral junction with a suture. The distal side of the urethra was also closed with a suture. The IUP was equilibrated at 20 mmHg by injecting a small volume of saline. After approximately

**TABLE 1**

<table>
<thead>
<tr>
<th>LDT</th>
<th>R₁</th>
<th>R₂</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td></td>
<td>−CH₃</td>
<td>1-(2-methoxyphenyl)-4-[2-(3- methoxyphenyl)ethyl]piperazine</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>−CH₃</td>
<td>1-(2-methoxyphenyl)-4-[2-(3,4-dimethoxyphenyl)ethyl]piperazine</td>
</tr>
<tr>
<td>8</td>
<td>−CH₂CH₃</td>
<td>−CH₃</td>
<td>1-(1,3-benzodioxol-5-ethyl)-4-ethoxyphenylpiperazine</td>
</tr>
<tr>
<td>66°</td>
<td></td>
<td>−CH₃</td>
<td>1-(2-methoxyphenyl)-4-hexylpiperazine</td>
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</table>

*From Chagas-Silva et al., 2014, with permission.*
30 minutes, IUP was increased by an i.v. administration of 1–100 μg·kg⁻¹ phenylephrine every 10 minutes. Alternatively, a single dose of 30 μg·kg⁻¹ phenylephrine was injected 10 minutes after the administration of 0.1 μg·kg⁻¹ LDT3 and LDT5 as a first evaluation of the pharmacological effect. Then full dose-response curves were constructed using LDT3 or LDT5 (0.01–3 μg·kg⁻¹ i.v.) or tamsulosin (0.001–1 μg·kg⁻¹ i.v.). Data were analyzed by nonlinear regression (GraphPad Prism 5.0; GraphPad Software) to determine the ED₅₀.

**Binding Assays with Native Receptors.** Rat brains were removed to obtain the hippocampus (5-HT₁A receptor) and cortex for antagonist ([³H]-prazosin) binding assays were performed using prazosin at concentrations ranging from 0.0001 to 0.1 μM. Binding was defined in the presence of 1 μM GTP (Chagas-Silva et al., 2014). The Kᵢ values for [³H]-8-OH-DPAT for 5-HT₁A receptors (0.7 nM) and of [³H]-ketanserin for 5-HT₂A receptors (1.7 nM) were previously estimated in our experimental conditions (Neves et al., 2010).

**Cell Growth Assays.** Human DU145 prostate cancer cells (75th–77th passage) and prostate cells from BPH patients (9th–11th passage; de Souza et al., 2011) were cultured in RPMI 1640 or DMEM.
respectively, supplemented with 10% fetal bovine serum, 1% sodium pyruvate, and 1% penicillin/streptomycin (37°C, 5% CO₂), until confluence. For cell growth assays, 5 × 10⁴ or 3 × 10⁵ cells/well (for DU145 and BPH, respectively) were seeded in 96-well plates and cultured in serum-free medium for 24 h, and then incubated for 48 h in medium containing 2.5% fetal bovine serum and 1 μM 5-HT or 3 μM phenylephrine, in the absence or presence of LDTs, BMY7378, or p-MPPF (fresh medium with drugs was added at 24 h). Cell growth was evaluated by counting of viable cells using Trypan blue as an exclusion dye or by the MTT assay. Data were expressed as the percentage of the control condition (vehicle).

**Statistical Analysis.** Otherwise indicated, data are expressed as means and S.D. The significance of the differences among two or more conditions was determined by Student’s t test or one-way analysis of variance (ANOVA), respectively. ANOVA was followed by post hoc Dunnett’s or Newman-Keuls test. The F values calculated were indicated in the legend of the tables and figures. Differences were considered statistically significant if P < 0.05.

**Results**

LDT3, LDT5, and LDT8 Have High Affinity for Native α₁A- and α₁D-Adrenoceptors, but Not for Off-Target Receptors. In assays using rat prostate, in which the subtype α₁A-adrenoceptor is the most important for contraction (Hiraoka et al., 1999), our results indicate that LDT3, LDT5, and LDT8 have high affinity for α₁A-adrenoceptors, with Kᵦ values ranging from 0.17 to 2.62 nM (Fig. 1A; Table 2), close to those for the anti-BPH drug tamsulosin and slightly smaller than the previous derivative LDT66 (Table 2).

In rat aorta, in which the main α₁-adrenoceptors responsible for contraction belong to the α₁D-subtype (Hussain and Marshall, 1997), treatment with N-phenylpiperazine derivatives also induced a shift of the phenylephrine concentration-response curves to the right, suggesting a surmountable antagonism (Fig. 1B). All LDTs showed high affinity for α₁D-adrenoceptors, with Kᵦ values in the low nanomolar range (Table 2), and the affinities of LDT5 and LDT8 for these receptors (Kᵦ = 0.59 and 0.18 nM, respectively) were significantly higher than that of the selective antagonist BMY7378 (Kᵦ = 3 nM; Table 2).

Analysis of the monophasic binding competition curves obtained with LDTs indicates that LDTs have lower affinity for α₁D-adrenoceptors than prazosin (Fig. 1C), with mean Kᵦ values ranging from 7 to 80 nM, compared with Kᵦ = 0.3 nM for prazosin (n = 3, P < 0.05). Moreover, test compounds Kᵦ values were also higher than their Kᵦ values for α₁D- and α₁A-adrenoceptors (P < 0.05). Indeed, LDT3 (Kᵦ = 80 nM, n = 4), LDT5 (Kᵦ = 10 nM, n = 5), and LDT8 (Kᵦ = 7 nM, n = 5) had 17- to 41-fold less affinity for the α₁D than for the α₁A-adrenoceptors (Table 2). In this experimental condition, tamsulosin showed a Kᵦ value of 5.9 nM for α₁A-adrenoceptors (Chagas-Silva et al., 2014), in agreement with previous reports (Williams et al., 1999; Pulito et al., 2000).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Compound</th>
<th>Control EC₅₀ (μM) [log EC₅₀ ± SD]</th>
<th>Treated EC₅₀ (μM) [log EC₅₀ ± SD]</th>
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<tr>
<td>α₁A</td>
<td>LDT3</td>
<td>0.24 [-6.62 ± 0.19]</td>
<td>1.2 [-5.92 ± 0.20]</td>
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<tr>
<td></td>
<td>LDT5</td>
<td>0.14 [-8.86 ± 0.27]</td>
<td>7.9 [-5.10 ± 0.37]</td>
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<tr>
<td></td>
<td>LDT8</td>
<td>0.25 [-6.60 ± 0.24]</td>
<td>14.8 [-4.83 ± 0.27]</td>
</tr>
<tr>
<td></td>
<td>LDT66</td>
<td>—</td>
<td>—</td>
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<tr>
<td>β₂</td>
<td>Tamsulosin</td>
<td>0.15 [-8.78 ± 0.23]</td>
<td>5.55 [-5.45 ± 0.24]</td>
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<td>LDT3</td>
<td>0.08 [-7.12 ± 0.23]</td>
<td>1.34 [-5.87 ± 0.23]</td>
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<td>LDT5</td>
<td>0.07 [-7.14 ± 0.09]</td>
<td>4.58 [-5.34 ± 0.14]</td>
</tr>
<tr>
<td></td>
<td>LDT66</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>β₃</td>
<td>BMY7378</td>
<td>0.009 (2)</td>
<td>0.59 (8)</td>
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</table>

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<th>α₅D</th>
<th>Compound</th>
<th>Kᵦ (nM) [SD]</th>
<th>Kᵦ (nM) [SD]</th>
<th>Kᵦ/Kᵦ (95% CI)</th>
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<tr>
<td>LDT3</td>
<td>1.12 (4)</td>
<td>1.73 (3)</td>
<td>1.53</td>
<td>(0.95 ± 0.07)***</td>
</tr>
<tr>
<td></td>
<td>(8.95 ± 0.19)***</td>
<td>(8.76 ± 0.19)***</td>
<td>(1.02-2.61)</td>
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<tr>
<td>LDT5</td>
<td>2.51 (4)</td>
<td>6.91 (3)</td>
<td>2.75</td>
<td>(8.60 ± 0.05)***</td>
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<tr>
<td></td>
<td>(8.16 ± 0.13)***</td>
<td>(6.68-4.50)***</td>
<td>(8.50-5.45)</td>
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<tr>
<td>LDT8</td>
<td>0.009 (2)</td>
<td>0.62 (3)</td>
<td>68.9</td>
<td>(11.05 ± 0.03)***</td>
</tr>
<tr>
<td></td>
<td>(9.12 ± 0.07)***</td>
<td>(45.9-99.8)***</td>
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<tr>
<td>LDT66</td>
<td>0.009 (2)</td>
<td>0.62 (3)</td>
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<td>(45.9-99.8)***</td>
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<td>5-HT</td>
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<td>0.62 (3)</td>
<td>68.9</td>
<td>(11.05 ± 0.03)***</td>
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<td>(45.9-99.8)***</td>
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**TABLE 2**

Affinity of N-phenylpiperazine derivatives for native rat α₁D- and α₁A-adrenoceptors

**TABLE 3**

Affinity of LDT3, LDT5, and LDT8 for rat 5-HT₁A receptors

Ki values were determined in competition-binding assays with the agonist [³H]-OH-DPAT (Kᵦ,high) or antagonist [³H]-p-MPPF (in the presence of high GTP; Kᵦ,low), using membrane preparations of rat hippocampus. pKi values (i.e., −log Kᵦ) were expressed as arithmetic means of n experiments. The Kᵦ,low/Kᵦ,high ratio is an estimate of the intrinsic activity toward 5-HT₁A receptors, where values significantly higher than 1 indicate agonist activity (5-HT, was used as an example in experiments). The pKi values were estimated using isomeric contraction assays of rat aorta stimulated with phenylephrine in the absence or presence of 50 nM (Kᵦ,low), or 10 nM (Kᵦ,high). BMY7378 (10 nM) was used as a selective antagonist of α₁D-adrenoceptors.

Log Kᵦ values were calculated individually.

F₁,33 = 57.10, P < 0.0001 for α₁A-adrenoceptors, ***P < 0.001 compared with tamsulosin (one-way ANOVA followed by a post hoc Dunnett’s test).

F₁,29 = 27.10, P < 0.0001 for α₁A-adrenoceptors, ***P < 0.001 compared with BMY7378 (one-way ANOVA followed by a post hoc Dunnett’s test).

Data from Chagas-Silva et al., 2014 used for comparison (with permission).

Calculated from pA₂ values by Schild regression of mean curves (Chagas-Silva et al., 2014) used for comparison (with permission).
We also evaluated the affinity of LDT3, LDT5, and LDT8 for the BPH off-target \( \alpha_2 \)-adrenoceptors and muscarinic receptors, using binding competition assays. LDTs showed \( K_i \) values in the micromolar range (0.2–108 \( \mu M \)), indicating a very low affinity for \( \alpha_2 \)-adrenoceptors and muscarinic receptors (Supplemental Table 1).

**LDT3 and LDT5 Are High-Affinity Antagonists of 5-HT\(_{1A} \) Receptors, with Low-Affinity for the Off-Target 5-HT\(_{2A} \).** Competition-binding experiments revealed that all test compounds had high affinity for 5-HT\(_{1A} \) receptors, with \( K_i \) values in the low nanomolar range for LDT3 and LDT5, and significantly lower for LDT8 (\( K_i = 9 \) pM, \( P < 0.05 \); Table 3). As the selectivity between 5-HT receptor subtypes is therapeutically relevant, we also measured the affinity of LDTs for the off-target 5-HT\(_{2A} \) receptor. All three LDTs had lower affinity for 5-HT\(_{2A} \) receptors (\( K_i = 70–389 \) nM) than for 5-HT\(_{1A} \) receptors, and 5-HT\(_{2A}/5-HT_{1A} \) ratios suggested high selectivity for 5-HT\(_{1A} \) (60- to 44,000-fold; Supplemental Table 2).

We also determined the intrinsic activity of our compounds toward 5-HT\(_{1A} \) receptors. For this purpose, we used the \( K_i \) ratio method, a functional binding assay that we recently described and compared with two other methods (GTP-shift and \([^{35}S]-GTPyS \) binding assay), for estimation of the intrinsic activity at the 5-HT\(_{1A} \) receptor (Noël et al., 2014). Using the \( K_i \) ratio method, the full agonist 5-HT showed a \( K_i \) ratio of 76.8. The results in Table 3 suggest that LDT3 and LDT5 are 5-HT\(_{1A} \) receptor antagonists (\( K_i \) ratio close to unity), whereas LDT8 had a \( K_i \) ratio value compatible with a partial agonist. Because antagonism at the 5-HT\(_{1A} \) receptor is supposed to be a prerequisite for efficacy of the type of multi-target BPH lead compound we aimed to develop, we interrupted the pharmacological characterization of LDT8 at this point.

**Fig. 2.** Inhibition of \( \alpha_1D \)-adrenoceptor- and 5-HT\(_{1A} \) receptor-dependent prostate cell growth by LDTs. Trypan blue exclusion assays were performed using prostate cells from BPH patients. BMY7378 (50 nM) and \( \beta \)-MPPF (50 nM, B) were used as selective antagonists of \( \alpha_1D \)-adrenoceptors (A) and 5-HT\(_{1A} \) receptors (B), respectively. PHE, phenylephrine. The mean number of cells in the control condition (vehicle) was 1.13 \( \times 10^5 \) cells. Data were expressed as mean and S.D. \( n = 3–4 \) different cell cultures using cells from three donors. Assays performed in triplicates. (A) \( F_{3,28} = 5.086, P = 0.0064 \). (B) \( F_{1,23} = 12.30, P < 0.0001 \). *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \) versus the agonists (one-way ANOVA followed by the post hoc Dunnnett’s test).

**Fig. 3.** Effect of LDT3 treatment on phenylephrine (PHE)-induced intracellular calcium elevation in Rnt-1 fibroblasts transfected with the human \( \alpha_{1D} \)-adrenoceptor. The sharp increase in the intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) induced by 100 \( \mu M \) PHE (A; vehicle) was attenuated by treatment with 10 and 100 nM LDT3 (B and C, respectively).
LDT3 and LDT5 Inhibit the Proliferation of Prostate Cells from BPH Patients. LDT3 and LDT5 behaved as high-affinity antagonists of rat α1D-adrenoceptors and 5-HT1A receptors (Tables 2 and 3); thus, we decided to verify whether these compounds had antiproliferative activity against prostate cells from BPH patients. To induce prostate cell proliferation in vitro, we used 3 μM of either phenylephrine or 5-HT, and the antagonists BMY7378 and p-MPPF (50 nM) were used as positive controls for α1D-adrenoceptors and 5-HT1A-receptor inhibition, respectively. The LDT concentration used (50 nM) corresponded to ~30- to 50-fold the estimated in vitro affinity of these compounds for α1D-adrenoceptors and 5-HT1A receptors. Counts of viable cells grown for 48 h in the presence of LTDs showed that both LDT3 and LDT5 inhibited BPH cell growth induced by phenylephrine and 5-HT, similarly to that observed using nontransformed BPH patient cells (Fig. 2). LDT3 and LDT5 also had a high potency at 1D-adrenoceptors (Table 2; Chagas-Silva et al., 2014). Similar effects of LTDs on BPH cells were obtained using the MTT assay (Fig. 2A) and p-MPPF (Fig. 2B). We did not observe clear cytotoxic effects when LTDs were used alone (data not shown). Note that tamsulosin produced only partial inhibition of phenylephrine-induced growth at 5 nM (Fig. 2A) and showed that both LDT3 and LDT5 inhibited BPH cell growth induced by phenylephrine and 5-HT, similarly to that observed using nontransformed BPH patient cells (Fig. 2), LDT3 and LDT5 also inhibited the 5-HT-stimulated growth of the prostate cancer cell line DU-145 (Supplemental Fig. 4). Interestingly, LDT8 stimulated DU-145 cell growth (Supplemental Fig. 4), which is compatible with the partial 5-HT1A agonist activity detected for this compound in binding assays (Table 3).

LDT3 and LDT5 Decrease Phenylephrine-Induced Calcium Elevation in Cells Overexpressing Human α1A- and α1D-Adrenoceptors. To confirm the antagonistic properties of LDT3 and LDT5 toward different human α1-adrenoceptor subtypes, we used a functional assay based on [Ca2+]i elevation in Rat-1 cells overexpressing human α1A-, α1D-, or α1B-adrenoceptors (Vázquez-Prado et al., 1997). In Rat-1 cells expressing α1D-adrenoceptors, stimulation with 100 μM phenylephrine typically induced a pronounced and transient increase in [Ca2+]i levels (Fig. 3A). In contrast, we observed a considerably less pronounced increase in [Ca2+]i after phenylephrine stimulation in cells incubated with LDT3 (Fig. 3, B and C) or LDT5. For these α1D-adrenoceptors, the mean IC50 (and SD) values of LDT3 and LDT5 were 8.5 ± 0.67 (n = 6) and 8.38 ± 0.06 (n = 4), respectively (Supplemental Fig. 5). The IC50 value of BMY7378 was 8.53 ± 0.08 (n = 3). LDT3 and LDT5 also had a high potency at α1A-adrenoceptors, with IC50 values of 7.53 ± 0.37 and 7.16 ± 0.51, respectively (n = 3), which were smaller than for tamsulosin (8.36 ± 0.33, n = 3, F2,6 = 6.718, P = 0.0294. P < 0.05 one-way ANOVA followed by Newman-Keuls test). In contrast, LDT3 and LDT5 had considerably lower affinity for α1B-adrenoceptors, with IC50 values for inhibition of [Ca2+]i
elevation of 6.10 ± 0.29 and 5.88 ± 0.34 (n = 3), respectively (P < 0.05 versus α1A, and P < 0.001 versus α1D-adrenoceptors). In this assay, the pIC_{50} value for tamsulosin was 8.8 ± 0.57 (n = 4). The overall ranking of potency of LDT3 and LDT5 at human α1-adrenoceptor subtypes was α1D > α1A > α1B (LDT3: F_{5,9} = 19.41, P = 0.0005; LDT5: F_{5,6} = 37.08, P = 0.0004; P < 0.01 one-way ANOVA, followed by Newman-Keuls test).

LDT3 and LDT5 Prevent Phenoxyline-Induced Increase in Intravesical Pressure, and Do Not Affect Basal Blood Pressure. As prostate contraction increases IUP (Akiyama et al., 1999), we investigated the effect of LDTs on rat IUP in vivo. Phenoxyline increased IUP in a dose-dependent manner, with an ED_{50} value of 7.5 μg.kg⁻¹ (Fig. 4A). Pretreatment with either LDT3 or LDT5 (0.1 μg.kg⁻¹, i.v.) prevented the phenoxyline-induced increase in IUP (30 μg.kg⁻¹), and LDT5 was more effective than LDT3 (Fig. 4B). In higher doses, both compounds fully blocked the phenoxyline effect. The mean ED_{50} values of LDT3 and LDT5 (Fig. 4C), 0.15 and 0.09 μg.kg⁻¹, respectively, were higher than the ED_{50} value of tamsulosin (0.007 μg.kg⁻¹).

Considering that hypotension is a classic adverse effect of α1-antagonists, we evaluated the effect of LDT3 and LDT5 on rat basal blood pressure. We observed a small reduction in basal blood pressure after treatment with 0.1 μg.kg⁻¹ LDT3 and LDT5 (mean and S.D.: -2.92 ± 2.6 and -2.13 ± 1.49%, respectively; n = 6), but this effect was not statistically different from that observed when saline was used as control (0.046 ± 6.5%; n = 5; P = 0.46). In addition, we further explored the effect of higher doses of LDT5, and only the dose of 100 μg.kg⁻¹, i.v., reduced significantly the basal blood pressure (Fig. 4D).

Discussion

Randomized controlled clinical trials have shown that blockage of prostatic α1-adrenoceptor is the most effective pharmacological management for relieving LUTS/BPH. For instance, both short- and long-term studies have shown that this pharmacological class improves the symptoms and the urinary flow rate (Lepor et al., 1996; Kirby et al., 2003; McConnell et al., 2003; Chapple, 2005; Oelke et al., 2013). Nevertheless, some patients may be unresponsive to α1A-adrenoceptor blockade (Kaplan, 2006), which limits the efficacy of α1A-adrenoceptor antagonists; in such cases, the risk of acute urinary retention is not reduced (McVary et al., 2011). The improvement of LUTS/BPH mediated by the association of 5-α reductase inhibitors with α1-adrenoceptor blockers as compared with monotherapy with α1-adrenoceptor blockers is only clearly observed after long-term therapy, whereas some adverse effects of 5-α reductase inhibitors may reduce patient compliance to treatment (McConnell et al., 2003; Chapple, 2005; Nickel, 2006; Tarle et al., 2009; Oelke et al., 2013). In this study, we show that the N-phenylpiperazine derivatives LDT3 and LDT5 inhibit rat prostate muscle contraction in vivo and human hyperplastic prostate cell growth in vitro.

Some diseases, including BPH, are multifactorial (Roehrborn, 2008), most likely requiring multi-target strategies to improve therapeutic efficacy (Morphy et al., 2004; Lu et al., 2012). For the clinical management of BPH, we hypothesized that targeting of α1D-adrenoceptors and 5-HT_{1A} receptors, in addition to α1A-adrenoceptor antagonism, could be particularly interesting because both receptors stimulate prostate cell growth (Dizeyi et al., 2004; Kojima et al., 2009a), α1D-adrenoceptors mRNA expression is increased in BPH (Kojima et al., 2009a), and nonprostatic α1D-adrenoceptors may contribute to bladder overactivity (Malloy et al., 1998; Michel, 2010; Kurizaki et al., 2011).

The α1A-adrenoceptor mediates human (Forray et al., 1994) and rat (Hiruoka et al., 1999) prostatic contraction (Michel and Vrydag, 2006). LDT3, LDT5, and LDT8 had high affinity for α1A-adrenoceptors in functional assays (Table 2). The affinities of LDT5 and LDT8 for rat α1A-adrenoceptors were similar to that of the clinically used anti-BPH agent tamsulosin (Table 2; Noble et al., 1997), and higher than the previous derivative LDT6.

Aside from their high affinity for rat and human α1A- and α1D-adrenoceptors, LDT3 and LDT5 also have low affinity for the off-target α1B subtype, in both species. α1-Adrenoceptor blockers are considered similar in efficacy to reduce LUTS/BPH, but they differ in tolerability (Michel, 2010; Kim et al., 2014). For instance, silodosin may be adequate to BPH patients receiving antihypertensive treatment (as this drug has little impact on blood pressure), whereas alfuzosin may be suitable for sexually active patients (as silodosin has the highest risk of ejaculatory dysfunction) (Chapple, 2005; Kim et al., 2014). Because human vascular expression of α1B-adrenoceptors increases in aging (Rudner et al., 1999), the low affinity of LDT3 and LDT5 for this receptor subtype may also reduce the risk of hypotension, a classic adverse effect of therapy with α1A-adrenoceptor antagonists (Jelski and Speakman, 2012). Moreover, some data suggest that tamsulosin could be more prone to induce high-grade intraoperative floppy iris syndrome (McVary et al., 2011; Chang et al., 2014).

Despite the fact that rat prostate does not surround urethra, it contributes to the increase of the IUP (Akiyama et al., 1999). Data from in vivo assays indicated clearly that LDT3 and LDT5 blocked the increase in rat IUP induced by phenylephrine (Fig. 4B), and LDT5 has an ED_{50} value of 0.09 μg.kg⁻¹ (Fig. 4C). In the same model, a similar dose of the previous derivative LDT66 (0.1 μg.kg⁻¹ i.v.) was about 20% less effective than LDT5 (data not shown). Therefore, our results suggest that LDT3 and LDT5 are capable of relaxing rat prostate. If translated to human prostate, we hypothesize that they could inhibit the dynamic component of BPH. Furthermore, we also showed that a similar dose of LDT3 and LDT5 (0.1 μg.kg⁻¹) did not affect rat basal blood pressure, which suggests uroselectivity at least for LDT5. Based on our results, it is possible that LDT5, in the doses used to reduce LUTS, would be neutral in relation to blood pressure.

The role of G protein–coupled receptors in cell growth has been investigated (revised in Liebmann, 2011). Keefel and colleagues (2000) showed in Chinese hamster ovary cells that the stimulation of transfected human α1D-adrenoceptor increases cell growth and extracellular signal-regulated kinase signaling. Moreover, it was shown that receptor stimulation induces proliferation of smooth muscle cells and fibroblasts from the adventitia of rat aorta, and the mechanism involved epidermal growth factor receptor transactivation (Zhang et al., 2004). In vivo, chronic stimulation of α1A-adrenoceptors induces rat prostatic hyperplasia involving transforming growth factor-β signaling (Kim et al., 2009). Actually,
transforming growth factor-β signaling has been considered as one of the mechanisms that contribute to human prostate enlargement (Descacaud et al., 2011).

α1D-Adrenoceptor mRNA have been shown in human hyperplastic prostate samples (Nasu et al., 1996; Kojima et al., 2006, 2009a; Morelli et al., 2014). Naftopidil, which has 3 and 7 times higher affinity for human α1D- than for α1A- and α1B-adrenoceptors, respectively (Takei et al., 1999), reduces prostate cell growth by arresting cell cycle at G1 phase (Kojima et al., 2009a,b). However, the presence of prostate α1D-adrenoceptor at protein level is still controversial, which warrants further investigation (Michel and Vrydag, 2006; Kojima et al., 2009b).

LDT3 and LDT5 inhibited the phenylephrine-induced growth of prostate cells from BPH patients (Fig. 2; Supplemental Fig. 3), and of DU-145 prostate cancer cells in a way qualitatively similar to BMY7378, which suggests the role of α1D-adrenoceptors (Supplemental Fig. 4). In our model, although LDT66 blocked the phenylephrine effect (DU-145) (P < 0.01), it also caused a slight proliferative effect when used alone (Chagas-Silva et al., 2014). Other α1-adrenoceptor antagonists with a quinazoline moiety also inhibit prostate cell growth in vitro; however, this effect is independent of α1-adrenoceptor and involves anoikis in prostate cells mediated by death receptors (reviewed in Kyprianou et al., 2009). Therefore, the mechanism of action involved in the antigrowth effect of the present N-phenylpiperazine derivatives, LDT3 and LDT5, depends on the blockade of the agonist action and differs from the effect of quinazoline drugs.

Another important signaling molecule that stimulates prostate cell growth is 5-HT, which is synthesized by neuroendocrine cells (Abrahamsson et al., 1986). 5-HT1A receptors stimulate the growth of some cell types such as fibroblasts (Abdel-Baset et al., 1992). Benign and malignant prostate tissues express 5-HT1A receptors, and mounting evidence suggests that these receptors stimulate prostate cell growth via Akt/mitogen-activated protein kinase pathway (Abdel-Baset et al., 1994; Dizeyi et al., 2004, 2011). Based on binding assays, LDT3 and LDT5 have high affinity for 5-HT1A receptors (Table 3). Importantly, LDT3 and LDT5 showed higher affinity for 5-HT1A receptors than LDT66 (Chagas-Silva et al., 2014) and naftopidil, another N-phenylpiperazine compound (Kᵢ = 107 nM; Borbe et al., 1991).

LDT3 and LDT5 also inhibited the 5-HT–induced BPH (and DU-145) cell growth in a similar manner to that of the selective 5-HT1A receptor antagonist p-MPPP (Fig. 2; Supplemental Fig. 3), in agreement with the antiproliferative effect of another 5-HT1A receptor antagonist (NAN 190) toward prostate cancer PC3 and DU-145 cell lines (Dizeyi et al., 2004). Moreover, whereas LDT3 and LDT5 blocked completely the agonist effect, LDT66 showed a partial inhibition (Chagas-Silva et al., 2014). In contrast, LDT8 behaved as a partial agonist of 5-HT1A receptors, in both binding and functional (cell growth) assays; thus, we discontinued the pharmacological testing of LDT8 for the purposes of multi-targeted anti-BPH therapy development.

Our data confirm that the N1-(2-methoxyphenyl)-N4-piperazine scaffold confers affinity for α1-adrenoceptors, as well as for 5-HT1A receptors (Glennon et al., 1988; Leopoldo et al., 2004; Chagas-Silva et al., 2014), and unveils the multi-target antagonist behavior of the N-phenylpiperazine derivatives described in this work. In addition, LDT5 showed higher affinity for α1A-, α1D-adrenoceptors, and 5-HT1A receptors than the previous derivative LDT66, which has a hexil substituion in the N₂-phenylpiperazine moiety (Chagas-Silva et al., 2014), and therefore, this compound showed an improved pharmacological profile at target receptors. This could suggest that the phenethyl auxophoric subunit present in LDT3 and LDT5 (R₁, Table 1), and absent in LDT66, is important for the interaction with amino acid residues in the before-mentioned receptors.

Also of note, the three LDTs tested in this study showed low affinity for off-target receptors, including α1B- and α2- adrenoceptors, as well as 5-HT2A and muscarinic receptors. Therefore, our data suggest that LDT3 and LDT5 are unlikely to cause the adverse effects associated with inhibition of important off-target receptors. We were also able to discard any interference of LDT3 and LDT5 (1 μM) with hERG K⁺ channel function (data not shown), whose blockade can elicit potentially fatal cardiac arrhythmias (Priest et al., 2008), which is the reason why this test is absolutely required for new drug approval by regulatory authorities (Bowes et al., 2012; Peters 2013).

Based on clinical data, current selective α1-adrenoceptor antagonists used to manage LUTS/BPH do not shrink prostate (McConnell et al., 2003; Kojima et al., 2009b). Overall, our results showed that LDT3 and LDT5 inhibit human hyperplastic prostate cell growth in vitro, while also relaxing prostate muscle, most probably by the multi-target antagonism of α1A-, α1D-adrenoceptors, and 5-HT1A receptors. Therefore, our working hypothesis is that the multi-target mechanism of action of the N-phenylpiperazine derivatives LDT3 and LDT5 could modify the course of the disease. If successfully translated to the clinic, these two important effects of LDTs could putatively modify the course of the disease by slowing prostate enlargement, and also alleviating LUTS/BPH. Thus, we propose that LDT3 and LDT5 are potential new lead compounds that could be of value for BPH treatment.

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Authorship Contributions

Participated in research design: Silva, Noël, Romeiro, García-Saínz.

Conducted experiments: Nascimento-Viana, Carvalho, Alcántara-Hernández, Chagas-Silva.

Contributed new reagents or analytic tools: Nasciutti, Souza.

Performed data analysis: Nascimento-Viana, Silva, Noël.

Wrote or contributed to the writing of the manuscript: Silva, Noël.

References


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References


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Supplemental Data

New multi-target antagonists of $\alpha_{1A}$-, $\alpha_{1D}$-adrenoceptors and 5-HT$_{1A}$ receptors reduce human hyperplastic prostate cell growth and the increase of intraurethral pressure


Journal of Pharmacology and Experimental Therapeutics
Supplemental Figure 1. Fourier transform infrared spectroscopy of LDT3 (upper), LDT5 (middle) and LDT8 (lower spectrum).
Supplemental Data

New multi-target antagonists of $\alpha_{1A}$-, $\alpha_{1D}$-adrenoceptors and 5-HT$_{1A}$ receptors reduce human hyperplastic prostate cell growth and the increase of intraurethral pressure


Journal of Pharmacology and Experimental Therapeutics
Supplemental Figure 2A. $^1$H-NMR mass spectrum of LDT3.
Supplemental Figure 2B. $^1$H-NMR mass spectrum of LDT5.
Supplemental Figure 2C. $^1$H-NMR mass spectrum of LDT8.
Supplemental Data

New multi-target antagonists of \(\alpha_{1A}, \alpha_{1D}\)-adrenoceptors and 5-HT\(_{1A}\) receptors reduce human hyperplastic prostate cell growth and the increase of intraurethral pressure


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Supplemental Methods

**Binding assays: off-target BPH receptors**

In all cases, the assay volume was 0.5 ml and the radioligand depletion at the end of the experiments was less than 15% with the exception of the assays with \[^3H\]-QNB in rat cortex preparation (around 40%) (Chagas-Silva et al., 2014).

For 5-HT\(_{2A}\) receptor assays, 150 \(\mu\)g cortical membrane protein were incubated with LTDs (10\(^{-10}\) – 10\(^{-4}\) M) in binding buffer containing 1 nM \[^3H\]-ketanserin and 100 nM prazosin, for 15 min at 37°C. Nonspecific binding was determined in the presence of 1 \(\mu\)M ketanserin.

For native \(\alpha_2\)-adrenoceptors, 150 \(\mu\)g cortical membrane protein were incubated with LTDs (10\(^{-8}\) – 10\(^{-4}\) M) in binding buffer containing 1 nM \[^3H\]RX821002, for 60 min at 30°C. Nonspecific binding was determined in the presence of 100 \(\mu\)M L-adrenaline bitartrate.

For native muscarinic receptors, 150 \(\mu\)g cortical membrane protein were incubated with 0.1 nM \[^3H\]QNB, 50 mM Tris-HCl in the presence of LTDs (10\(^{-6}\) – 10\(^{-3}\))
M), at 25°C for 60 min. Atropine sulphate (10 μM) was used to determine non-specific binding (Chagas-Silva et al., 2014).

Statistical analysis

Otherwise indicated, data are expressed as means and SD. The significance of the differences among two or more conditions was determined by Student’s t test or one-way analysis of variance (ANOVA) followed by post hoc Dunnett’s test, respectively.
Supplemental Table 1. Affinity of LDT derivatives for native rat α₂-adrenoceptors and muscarinic receptors.

<table>
<thead>
<tr>
<th>Compound (n)</th>
<th>α₂-adrenoceptors</th>
<th>muscarinic receptors</th>
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<tbody>
<tr>
<td></td>
<td>log IC₅₀ ± SD (M)</td>
<td>Kᵢ (µM)</td>
</tr>
<tr>
<td>LDT3 (4)</td>
<td>-5.97 ± 0.18</td>
<td>0.93</td>
</tr>
<tr>
<td>LDT5 (3)</td>
<td>-6.53 ± 0.09**</td>
<td>0.24</td>
</tr>
<tr>
<td>LDT8 (4)</td>
<td>-6.22 ± 0.24</td>
<td>0.55</td>
</tr>
<tr>
<td>LDT66a (3)</td>
<td>-5.92 ± 0.13ᵇ</td>
<td>0.81</td>
</tr>
<tr>
<td>yohimbine (2)</td>
<td>-6.76 ± 0.07</td>
<td>0.12</td>
</tr>
<tr>
<td>pirenzepine (2)</td>
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<table>
<thead>
<tr>
<th></th>
<th>log IC₅₀ ± SD (M)</th>
<th>Kᵢ (µM)</th>
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IC₅₀ values (expressed as mean ± SD) were calculated by nonlinear regression of data from binding competition assays using radiolabelled antagonists of α₂-adrenoceptors ([³H]RX-821002) and muscarinic receptors ([³H]-QNB). Yohimbine and pirenzepine were used as positive controls for α₂-adrenoceptor and muscarinic receptor antagonism, respectively. Kᵢ values were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973), considering Kᵗ values of 2.05 nM for [³H]RX-821002 (Chagas-Silva et al., 2014) and 0.05 nM for [³H]-QNB (Luthi and Wolfe, 1984). Experiments were performed in triplicates. a from Chagas-Silva et al., 2014 with permission; b n = 5.

F₃,₁₂ = 9.347, P = 0.0018 for α₂-adrenoceptors. ** P < 0.01 vs. LDT3 (one way ANOVA followed by post hoc Dunnett’s test)

F₃,₁₀ = 17.56, P = 0.0003 for muscarinic receptors. *P < 0.05 vs. LDT3 and LDT8 (one way ANOVA followed by post hoc Dunnett’s test)
Supplemental Table 2. Affinity and selectivity of LDTs towards native rat 5-HT receptors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (M) (n)</th>
<th>$K_i$ (M) (n)</th>
<th>Selectivity for 5-HT$<em>{1A}$ (5-HT$</em>{2A}$/5-HT$_{1A}$ $K_i$ ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[log IC$_{50}$ ± SD (M)]</td>
<td>[log IC$_{50}$ ± SD (M)]</td>
<td></td>
</tr>
<tr>
<td>LDT3</td>
<td>$1.12 \times 10^{-9}$ (4)</td>
<td>$7.08 \times 10^{-9}$ (3)</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>[-8.56 ± 0.07] ***</td>
<td>[-7.15 ± 0.38] #</td>
<td></td>
</tr>
<tr>
<td>LDT5</td>
<td>$2.51 \times 10^{-9}$ (4)</td>
<td>$3.89 \times 10^{-7}$ (3)</td>
<td>155</td>
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<tr>
<td></td>
<td>[-8.21 ± 0.05] ***</td>
<td>[-6.41 ± 0.03] #</td>
<td></td>
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<tr>
<td>LDT8</td>
<td>$8.85 \times 10^{-12}$ (2)</td>
<td>$3.89 \times 10^{-7}$ (3)</td>
<td>43,949</td>
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<tr>
<td></td>
<td>[-10.66 ± 0.03]</td>
<td>[-6.41 ± 1.21] **</td>
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<td>LDT66$^a$</td>
<td>$5.9 \times 10^{-9}$ (4)</td>
<td>$1.78 \times 10^{-6}$ (3)</td>
<td>300</td>
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<tr>
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<td>[-7.93 ± 0.40] ***</td>
<td>[-5.57 ± 0.28] #</td>
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</table>

Data were obtained using binding competition assays with the radioligands [$^3$H]-8-OH-DPAT (5-HT$_{1A}$ receptor) and [$^3$H]-ketanserin (5-HT$_{2A}$ receptor). $K_i$ values were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Experiments were performed in triplicates. $^a$ from Chagas-Silva et al., 2014 with permission. $F_{3,10} = 72.18$, $P < 0.0001$ for 5-HT$_{1A}$ receptors. *** $P < 0.001$ compared to LDT 8 (one-way ANOVA followed by a post hoc Dunnett’s test). ** $P < 0.01$, # $P < 0.001$ for 5-HT$_{2A}$ versus 5-HT$_{1A}$ receptors (Student’s $t$ test).
Supplemental Figure 3. Inhibition of human hyperplastic prostate cell growth by LDT3 and LDT5.

In these cells, proliferation (estimated by the MTT assay) induced by phenylephrine (A, PHE) or 5-HT (B) is mainly due to activation of $\alpha_{1D}$-adrenoceptors and 5-HT$_{1A}$ receptors, respectively. BMY 7378 and p-MPPF ($\alpha_{1D}$-adrenoceptors and 5-HT$_{1A}$ antagonists, respectively) were used as controls. Data are expressed as mean ± SD of 5 independent experiments performed in quintuplicates using three different cultures (see Methods).

$F_{7,32} = 7.558$, $P < 0.0001$ for $\alpha_{1D}$-adrenoceptor. $F_{7,32} = 5.221$, $P = 0.0005$ for 5-HT$_{1A}$ receptor. ***$P < 0.001$ vs. agonist alone. One-way analysis of variance (ANOVA) followed by the post hoc Dunnett’s test.
Supplemental Figure 4. Inhibition of the growth of human DU-145 prostate cancer cells by LDT3, LDT5 and LDT8. Growth was estimated by MTT assay, and BMY 7378 (50 nM) and p-MPPF (50 nM) were used as selective antagonists of α₁D-adrenoceptors and 5-HT₁A receptors, respectively. In these cells, proliferation induced by phenylephrine (A, PHE) or 5-HT (B) is mainly due to activation of α₁D-adrenoceptors and 5-HT₁A receptors, respectively. Data are expressed as mean ± SD of 3-4 independent experiments performed in triplicates.

F₉₆₁ = 8.002, P < 0.0001 for α₁D-adrenoceptor. F₉₆₁ = 5.394, P < 0.0001 for 5-HT₁A receptor. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the agonists (one-way ANOVA followed by the post hoc Dunnett’s test).
Supplemental Figure 5. LDT3 and LDT5 inhibit the increase of intracellular Ca\(^{2+}\) in rat-1 cells transfected with α\(_1\)-adrenoceptor subtypes. The increase of intracellular Ca\(^{2+}\) [Ca\(^{2+}\)]\(_i\) was induced by 100 µM phenylephrine. Antagonists were incubated for 100 sec before the addition of the agonist. Tamsulosin (n=3) and BMY7378 (n=3) were used as controls. LDT3 n=6 (α\(_{1D}\)), n=3 (α\(_{1A}\), α\(_{1B}\)). LDT5 n=4 (α\(_{1D}\)), n=3 (α\(_{1A}\), α\(_{1B}\)). Note that the error bars used here are the SEM (instead of SD) of the means.