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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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-dimethylthiazol-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$_s$ 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 µg 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
and 5-HT1A receptors (Chagas-Silva et al., 2014). In this work, we hypothesized that not only 1A-adrenoceptor antagonists such as tamsulosin, or the uroselective 1A-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 1A-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 1A-adrenoceptor blockers are sheer prostate size and detrusor muscle contraction (McVary et al., 2011; Oelke et al., 2013).

The possibility that 1D-adrenoceptors also might play a role in the pathogenesis of BPH has been suggested. According to data obtained by RINase protection, in situ hybridization or reverse-transcription polymerase chain reaction assays, the expression level of 1A-, 1B-, and 1D-adrenoceptor mRNA differs among hyperplastic prostate samples; however, the prostatic expression of 1D-adrenoceptor mRNA is frequently increased in such condition (Nasu et al., 1996; Kojima et al., 2006, 2009a). Some reports have suggested that 1D-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., 2009a). However, it should be mentioned that detection of the 1A-adrenoceptor protein is controversial due to the lack of highly selective antibodies validated under stringent conditions (Pradadarcheep et al., 2009; Böhmer et al., 2014). The discrepancies in the reported mRNA and protein expression in the prostate warrant that caution needs to be exercised until additional data are obtained. Human bladder also expresses 1D-adrenoceptors (Malloy et al., 1998), and both the expression and function increase due to bladder outlet obstruction, both in rats and humans (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 1A-adrenoceptors (Hieble, 2011), so that we hypothesized that not only 1D-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 prostatic 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 1A-, 1D-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 1A- and 1D-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.

### 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 DFBC-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.

**Test Compounds.** The N-phenylpiperazine derivatives LDT3, LDT5, and LDT8 (Table 1) were synthesized by LADETER (Universidade Católica de Brasilia, Brasilia, Brazil), and were available in the monohydrochloride form, as previously described for other N-phenylpiperazine derivatives (Romeiro et al., 2011). Fourier transform infrared spectroscopy spectra (Supplemental Fig. 1) were recorded on a Spectrum BX spectrometer (PerkinElmer, Waltham, MA); 1H-NMR (300 and 500 MHz, CDCl3) (Supplemental Fig. 2) and 13C-NMR (75 and 125 MHz, CDCl3) spectra were recorded on plus Varian (7.05 T) and Bruker Avance DRX500 and DRX300 spectrometers; and the mass spectra were recorded on a Shimadzu LCMS IT-TOF spectrometer. The spectrometric analysis revealed the presence of only one compound in each sample.

**Drugs and Radioligands.** Prazosin hydrochloride, pargyline hydrochloride, 5-hydroxytryptamine hydrochloride (5-HT), acetylcholine hydrobromide (8-OH-DPAT), 2-methoxy idazoxan (RX821002), 1-(2-methoxyphenyl)-N-(2-pyridinyl)benzamide dihydrochloride ([3H]-prazosin (85 Ci/mmol), [3H]-ketanserin (60 Ci/mmol), [3H]-8-OH-DPAT (187 Ci/mmol), and [3H]-propranolol hydrochloride, 4-fluoro-N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylbenzamidedihydrochloride (p-MPPF), 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride (BMY7378), 8-hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT), 2-methoxy idoxan (RX821002), 3-quinuclidinyl benzilate (QNB), tamsulosin hydrochloride, ketanserin tartrate, polyethyleneimine, atropine sulfate, and GTP were purchased from Sigma-Aldrich (St. Louis, MO). [3H]-prazosin (85 Ci/mmol), [3H]-ketanserin (60 Ci/mmol), [3H]-8-OH-DPAT (187 Ci/mmol), and [3H]-p-MPPF (74.2 Ci/mmol) were obtained from PerkinElmer. [3H]-RX821002 (60 Ci/mmol) and [3H]-3-quinuclidinyl benzilate ([3H]-QNB, 250 Ci/mmol) were obtained from Amersham (Challont, UK).

**Isometric Contraction Assays.** Rat prostate and thoracic aorta were removed, cleaned, and cut into 10-mm strips (prostate) or 3-mm rings (aorta). Isometric contraction assays were performed as described previously (Chagas-Silva et al., 2014). Samples were placed in an organ bath containing a physiologic solution (prostate, mM): NaCl 138, KCl 5.7, CaCl2 1.8, NaH2PO4 0.36, NaHCO3 15 and glucose 5.5; [aorta, mM]: NaCl 122, KCl 5, NaHCO3 15, glucose 11.5, MgCl2 1.25, CaCl2 1.25 and KH2PO4 1.25 (95% O2 and 5% CO2, 37°C). Prostate
and denuded aorta segments were preloaded (60 minutes) with 10 or 20 mM, respectively, and washed, twice. Tissues were contracted with 1 mM 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 mM propranolol, before and after incubation for 60 minutes with the test compounds (10 or 50 mM), 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, Bella Vista, Australia). 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.

### Intracellular Ca^{2+} Measurement.

The effect of LDTs on human α1-adrenoceptor subtypes was determined by measuring intracellular Ca^{2+} in rat-1 fibroblasts stably expressing α1A-, α1B-, or α1D-adrenoceptors (Vázquez-Prado et al., 1997). These cells were cultured in high-glucose Dulbecco’s 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% CO2 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.

### Table 1

<table>
<thead>
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<th>LDT</th>
<th>R_{1}</th>
<th>R_{2}</th>
<th>Name</th>
</tr>
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<tr>
<td>3</td>
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<td>–CH_{3}</td>
<td>1-(2-methoxyphenyl)-4-[2-(3-methoxyphenyl)ethyl]piperazine</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>–CH_{3}</td>
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</tr>
<tr>
<td>8</td>
<td></td>
<td>–CH_{2}CH_{3}</td>
<td>1-(1,3-benzodioxol-5-ylethyl)-4-ethoxyphenylpiperazine</td>
</tr>
<tr>
<td>66</td>
<td>(CH_{2})<em>{3}CH</em>{3}</td>
<td>–CH_{3}</td>
<td>1-(2-methoxyphenyl)-4-hexylpiperazine</td>
</tr>
</tbody>
</table>

*From Chagas-Silva et al., 2014, with permission.*

**Measurement.** The effect of LDTs on human α1-adrenoceptor subtypes was determined by measuring intracellular Ca^{2+} in rat-1 fibroblasts stably expressing α1A-, α1B-, or α1D-adrenoceptors (Vázquez-Prado et al., 1997). These cells were cultured in high-glucose Dulbecco’s 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% CO2 atmosphere.

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**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).

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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.
30 minutes, IUP was increased by an i.v. administration of 1–100 
\( \mu g \) kg\(^{-1} \) phenylephrine every 10 minutes. Alternatively, a single dose of 30 \( \mu g \) kg\(^{-1} \) phenylephrine was injected 10 minutes after the administra-
tion of 0.1 \( \mu g \) kg\(^{-1} \) LDT3 and LDT5 as a first evaluation of the pharma-
cological effect. Then full dose-response curves were con-
structed using LDT3 or LDT5 (0.01–3 \( \mu g \) kg\(^{-1} \) i.v.) or tamsulosin (0.001–0.1 \( \mu g \) kg\(^{-1} \) i.v.). Data were analyzed by nonlinear regression (GraphPad Prism 5.0; GraphPad Software) to determine the ED\(_{50}\).

### Binding Assays with Native Receptors.

Rat brains were removed to obtain the hippocampus (5-HT\(_{1A}\) receptor) and cortex (5-HT\(_{1A}\), \( \alpha_2 \)-adrenoceptor and muscarinic receptors; Supplemental Methods) and stored in liquid nitrogen. Hippocampal and cortical membrane samples were prepared, as previously described (Neves et al., 2010). After incubation, binding samples were diluted (3 times in 4 ml) in ice-cold 5 mM Tris-HCl buffer (pH 7.4) and subjected to rapid filtration under vacuum using glass fiber filters (GMF 3; Filtrak, Thermalbad Wiesenbad, Germany) presoaked in 0.5% polyethylene-
mine (\( ^{3} \)H)-ketanserin, \( ^{3} \)H)-8-OH-DPAT, and \( ^{3} \)H]-RX821002 assays) or binding buffer (\( ^{3} \)H]-p-MPPF and \( ^{3} \)H]-prazosin assays). Radioactivity 
was determined using a Tri-Carb B2810 TR liquid scintillation counter (PerkinElmer). All assays were performed in triplicate. 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 \( ^{3} \)H]-prazosin in the rat liver preparation (35%). Ideally, radioligand depletion should be seized to less than 10% (Hulme and Trevethick, 2010), so that we have to consider a possible technical limitation on the precision of the affinity estimation, at least for the data with \( ^{3} \)H]-prazosin in the rat liver preparation, the assay used for labeling the off-target \( \alpha_2B \)-adrenoceptors. Nevertheless, the \( pK_i \) value of \( ^{3} \)H]-prazosin for \( \alpha_2B \)-adrenoceptors was 9.29 (0.51 nM), which was close to the value previously reported (\( pK_i \) 9.98 ± 0.27; Ohmura and Muramatsu, 1995).

For 5-HT\(_{1A}\) receptor assays, 50 \( \mu g \) membrane protein was incubated with LDTs (10\(^{-12}\)–10\(^{-6} \) M) in binding buffer containing 1 nM \( ^{3} \)H]-8-OH-DPAT (agonist), 1 mM CaCl\(_2\), 1 mM MnCl\(_2\), and 10 \( \mu M \) pargyline (for 15 minutes at 37°C) or 0.5 nM \( ^{3} \)H]-p-MPPF (agonist) and 1 mM GTP (45 minutes at 37°C). Nonspecific 

binding was determined in the presence of 10 \( \mu M \) 5-HT. The intrinsic activity of LDTs at 5-HT\(_{1A}\) receptors was determined, as described by Assié et al. (1999), using the dissociation constants (\( K_i \)) of the LDTs obtained for agonist (\( ^{3} \)H]-8-OH-DPAT) binding (\( K_i \)\(_{\text{High}} \)) and for antagonist (\( ^{3} \)H]-p-MPPF) binding in the presence of a high concentration of GTP (\( K_i \)\(_{\text{Low}} \)). \( K_i \)\(_{\text{Low}}/K_i \)\(_{\text{High}} \) values higher than 1.0 indicate agonism, values close to 1.0 suggest antagonism, and values lower than 1.0 indicate inverse agonism (Noél et al., 2014).

Rat livers (\( \alpha_2 \)-adrenoceptors) were minced in ice-cold 5 mM Tris (pH 7.4) containing 0.25 M sucrose and 1 mM EGTA, and then homogenized twice in 50 mM Tris HCl (pH 7.4) containing 100 mM NaCl and 2 mM EDTA (1.6, w/v), using an Ultra Turrax homogenizer. Liver homogenates were filtered through four layers of gauze and centrifuged at 5000 \( \times \) g, for 20 minutes at 4°C. Supernatants were ultracentrifuged at 100,000 \( \times \) g, for 60 minutes at 4°C, and final pellets were diluted in 5 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose (Michel et al., 1994). The protein content was determined, according to Lowry et al. (1951), using bovine serum albumin as a standard. Then 150 \( \mu g \) liver membrane protein was incubated with test compounds (10\(^{-9}\)–10\(^{-5} \) M) and 0.1 nM \( ^{3} \)H]-prazosin, in binding 

buffer containing 1 mM EDTA, for 45 minutes at 30°C. Nonspecific 

binding was defined in the presence of 1 \( \mu M \) prazosin. Alternatively, 
saturation assays were performed using prazosin at concentrations 

ranging from 0.001 to 1.0 \( \mu M \) (Chagas-Silva et al., 2014).

The binding assays for the \( \alpha_2 \)-adrenoceptors, 5-HT\(_{1A}\), and musca-
rinic receptors are described in Supplemental Material.

### Analysis of Binding Assays.

Data were analyzed by computerized nonlinear regression of untransformed data (GraphPad Prism 5.0; GraphPad Software), to estimate the IC\(_{50}\) of test compounds or radioligand \( K_i \) values. Dissociation constants (\( K_i \)) were calculated using the Cheng and Prusoff equation (Cheng and Prusoff, 1973).
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 × 10^4 or 3 × 10^5 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% sodium phenylpiperazine derivatives for native rat 1B-adrenoceptors (Hiraoka et al., 1999), our results indicate that LDT3, LDT5, and LDT8 have high affinity for native α1D- and α1A-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 K_B 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 K_B values in the low nanomolar range (Table 2), and the affinities of LDT5 and LDT8 for these receptors (K_B = 0.59 and 0.18 nM, respectively) were significantly higher than that of the selective antagonist BMY7378 (K_B = 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 K_i values ranging from 7 to 80 nM, compared with K_i = 0.3 nM for prazosin (n = 3, P < 0.05). Moreover, test compounds K_i values were also higher than their K_B values for α1D- and α1A-adrenoceptors (P < 0.05). Indeed, LDT3 (K_i = 80 nM, n = 4), LDT5 (K_i = 10 nM, n = 5), and LDT8 (K_i = 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 K_i 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 2**

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

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Compound</th>
<th>Control EC_50 (μM)</th>
<th>Treated EC_50 (μM)</th>
<th>log K_i SD (M)</th>
<th>K_i (nM)</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>
<td>−8.58 ± 0.29***</td>
<td>2.62</td>
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<td>LDT5</td>
<td>0.14 [−6.86 ± 0.27]</td>
<td>7.9 [−5.10 ± 0.37]</td>
<td>−9.74 ± 0.35</td>
<td>0.18</td>
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<td>LDT8</td>
<td>0.25 [−6.60 ± 0.24]</td>
<td>14.8 [−4.83 ± 0.27]</td>
<td>−9.76 ± 0.32</td>
<td>0.17</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>LDT66</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>3.4</td>
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</tbody>
</table>

For α1A-adrenoceptors, the EC_50 and K_i values were estimated using isomeric contraction assays of rat prostate stimulated with phenylephrine, in the absence or presence of 10 nM antagonist. Tamsulosin (10 nM) was used as control.

For α1D-adrenoceptors, EC_50 and K_i values were estimated using isomeric contraction assays of rat aorta stimulated with phenylephrine in the absence or presence of 50 nM (3), or 10 nM (3) antagonist. BMY7378 (10 nM) was used as a selective antagonist of α1D-adrenoceptors.

log K_i values were calculated individually.

For α1A-adrenoceptors, EC_50 and K_i values were estimated using isomeric contraction assays of rat aorta stimulated with phenylephrine in the absence or presence of 50 nM (3), or 10 nM (3) antagonist. BMY7378 (10 nM) was used as a selective antagonist of α1D-adrenoceptors.

log K_i values were calculated individually.

For α1D-adrenoceptors, EC_50 and K_i values were estimated using isomeric contraction assays of rat aorta stimulated with phenylephrine in the absence or presence of 50 nM (3), or 10 nM (3) antagonist. BMY7378 (10 nM) was used as a selective antagonist of α1D-adrenoceptors.

For α1A-adrenoceptors, the EC_50 and K_i values were estimated using isomeric contraction assays of rat aorta stimulated with phenylephrine in the absence or presence of 50 nM (3), or 10 nM (3) antagonist. BMY7378 (10 nM) was used as a selective antagonist of α1D-adrenoceptors.

log K_i values were calculated individually.

For α1D-adrenoceptors, EC_50 and K_i values were estimated using isomeric contraction assays of rat aorta stimulated with phenylephrine in the absence or presence of 50 nM (3), or 10 nM (3) antagonist. BMY7378 (10 nM) was used as a selective antagonist of α1D-adrenoceptors.

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log K_i values were calculated individually.
We also evaluated the affinity of LDT3, LDT5, and LDT8 for the BPH off-target α2-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 α2-adrenoceptors and muscarinic receptors (Supplemental Table 1).

**LDT3 and LDT5 Are High-Affinity Antagonists of 5-HT1A Receptors, with Low-Affinity for the Off-Target 5-HT2A.** Competition-binding experiments revealed that all test compounds had high affinity for 5-HT1A 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-HT2A receptor. All three LDTs had lower affinity for 5-HT2A receptors (\( K_i = 70–389 \) nM) than for 5-HT1A receptors, and 5-HT2A/5-HT1A ratios suggested high selectivity for 5-HT1A (60- to 44,000-fold; Supplemental Table 2).

We also determined the intrinsic activity of our compounds toward 5-HT1A 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 [\( ^{35}S \)]-GTP\( \gamma \)S binding assay), for estimation of the intrinsic activity at the 5-HT1A 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-HT1A 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-HT1A 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](https://example.com/fig2.jpg)

*Fig. 2.* Inhibition of \( \alpha_{1D} \)-adrenoceptor- and 5-HT1A receptor-dependent prostate cell growth by LDTs. Trypan blue exclusion assays were performed using prostate cells from BPH patients. BMY7378 (50 nM) and \( \rho \)-MPPF (50 nM, B) were used as selective antagonists of \( \alpha_{1D} \)-adrenoceptors (A) and 5-HT1A 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) \( F_{2,8} = 5.086, P = 0.0044 \). (B) \( F_{2,21} = 12.30, P < 0.0001 \). **P < 0.05, ***P < 0.01, and ****P < 0.001 versus the agonists (one-way ANOVA followed by the post hoc Dunnett’s test).

![Fig. 3](https://example.com/fig3.jpg)

*Fig. 3.* Effect of LDT3 treatment on phenylephrine (PHE)-induced intracellular calcium elevation in Rat-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 with LDT8 (50 nM). We did not observe clear cytotoxic effects when LDTs were used alone (data not shown). Note that tamsulosin produced only partial inhibition of phenylephrine-induced growth at 5 nM (Fig. 2A), a concentration ~50-fold higher than the Ki we reported for the α1D-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 pIC50 (and SD) values of LDT3 and LDT5 were 8.31 ± 0.67 (n = 6) and 8.38 ± 0.06 (n = 4), respectively (Supplemental Fig. 5). The pIC50 value of BMY7378 was 8.53 ± 0.08 (n = 3). LDT3 and LDT5 also had a high potency at α1A- and α1B-adrenoceptors, with pIC50 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; F,2.6 = 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 pIC50 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 pIC50 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: F2,9 = 19.41, P = 0.0005; LDT5: F2,6 = 37.08, P = 0.0004; P < 0.01 one-way ANOVA, followed by Newman-Keuls test).

LDT3 and LDT5 Prevent Phenylephrine-Induced Increase in Intraurethral 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. Phenylephrine increased IUP in a dose-dependent manner, with an ED50 value of 7.5 μg.kg⁻¹ (Fig. 4A). Pretreatment with either LDT3 or LDT5 (0.1 μg.kg⁻¹, i.v.) prevented the phenylephrine-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 phenylephrine effect. The mean ED50 values of LDT3 and LDT5 (Fig. 4C), 0.15 and 0.09 μg.kg⁻¹, respectively, were higher than the ED50 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 blockade 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). However, α1A-adrenoceptor or uroselective α1-adrenoceptor antagonists show better tolerability (Chapple, 2005; McVary et al., 2011; 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 5α 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 5α 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-HT1A receptors, in addition to α1A-adrenoceptor antagonism, could be particularly interesting because both receptors stimulate prostate cell growth (Dizey et al., 2004; Kojima et al., 2009a), α1D-adrenoceptor 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 LDT66.

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 α1-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 ED50 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). Keffel 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 α1-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 (Descaeud 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- 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 (revised 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 blockage 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 multitarget antagonist behavior of the N-phenylpiperazine derivatives described in this work. In addition, LDT5 showed higher affinity for α₁A- and α₁D-adrenoceptors, and 5-HT₁A receptors than the previous derivative LDT66, which has a hexil substitution 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-Sáinz.
Conducted experiments: Nascimento-Viana, Carvalho, Alcântara-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.

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675–683.

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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 $[^3]$H-$QNB$ in rat cortex preparation (around 40%) (Chagas-Silva et al., 2014).

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

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

For native muscarinic receptors, 150 μg cortical membrane protein were incubated with 0.1 nM $[^3]$H[QNB, 50 mM Tris-HCl in the presence of LTDs ($10^{-6}$ – $10^{-3}$ M).
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>
</tr>
</thead>
<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>
<td>---</td>
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</tr>
</tbody>
</table>

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 K_d 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.

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

Data were obtained using binding competition assays with the radioligands [³H]-8-OH-DPAT (5-HT$_{1A}$ receptor) and [³H]-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 α1D-adrenoceptors and 5-HT1A receptors, respectively. BMY 7378 and p-MPPF (α1D-adrenoceptors and 5-HT1A 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).

F7,32 = 7.558, P < 0.0001 for α1D-adrenoceptor. F7,32 = 5.221, P = 0.0005 for 5-HT1A 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.

F₉,₆₁ = 8.002, P < 0.0001 for α₁D-adrenoceptor. F₉,₆₁ = 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 $\alpha_1$-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 ($\alpha_{1D}$), n=3 ($\alpha_{1A}$, $\alpha_{1B}$). LDT5 n=4 ($\alpha_{1D}$), n=3 ($\alpha_{1A}$, $\alpha_{1B}$). Note that the error bars used here are the SEM (instead of SD) of the means.
References