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Title Page

New multi-target antagonists of α_{1A}-, α_{1D}-adrenoceptors and 5-HT_{1A} receptors reduce human hyperplastic prostate cell growth and the increase of intraurethral pressure

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Running title page.

Running title:

New multi-target drugs as potential leads for BPH treatment

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- Abstract: 253 words
- Introduction: 732 words
- Discussion: 1582 words

Abbreviations list:

5-HT	5-Hydroxytryptamine (serotonin)
BPH	Benign prostatic hyperplasia
IUP	Intraurethral pressure
LUTS	Lower urinary tract symptoms
PHE	Phenylephrine
TGF-β	transforming growth factor β
TLC	Thin-layer chromatography

Section: Drug Discovery and Translational Medicine

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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 α_{1A}-adrenoceptor antagonists, is helpful for many patients but insufficient for others, and recent reports suggest that stimulation of α_{1D}-adrenoceptors and 5-HT_{1A} receptors contribute to cell proliferation. Here, 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 α_1-adrenoceptors subtypes. The anti-proliferative effect of LDTs on prostate cells from BPH patients was evaluated by viable cell counting and MTT assays. We also determined LDTs effects on rat intraurethral and arterial pressure. LDT3 and LDT5 are potent antagonists of α_{1A}-, α_{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 induced by phenylephrine at doses (ED_{50} of 0.15 and 0.09 $\mu g.kg^{-1}$, 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.

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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). Amongst 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).

α_{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 α_1-adrenoceptor mRNA content (Nasu et al., 1996).

Both the American and European Urological Associations consider α_1-adrenoceptor antagonism as an appropriate pharmacological treatment to control moderate to severe LUTS/BPH (McVary et al., 2011; Oelke et al., 2013). Nevertheless, the introduction of α_{1A}-adrenoceptor antagonists such as tamsulosin, or the uroselective α_1-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 α_{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 α_{1A}-adrenoceptors blockers are sheer prostate size and detrusor muscle contraction (McVary et al., 2011; Oelke et al., 2013).

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The possibility that α_{1D}-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 RT-PCR assays, the expression level of α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors mRNA differs among hyperplastic prostate samples; however, the prostatic expression of α_{1D}-adrenoceptor mRNA is frequently increased in such condition (Nasu et al., 1996; Kojima et al., 2006; 2009a). Some reports have suggested that α_{1D}-adrenoceptors blockade may improve BPH treatment by inhibiting prostate cell growth *in vitro* and *in vivo* (Kojima et al., 2009a). On the other hand, tamsulosin, one of the most widely used drug for BPH treatment, is not as effective in this model (Kojima et al., 2009a). However, it should be mentioned that detection of the α_1-adrenoceptor protein is controversial due to the lack of highly selective antibodies validated under stringent conditions (Pradidarcheep 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 α_{1D}-adrenoceptors (Malloy et al., 1998) and both the expression and function increase due to bladder outlet obstruction, both in rat and man (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 α_{1A}-adrenoceptors (Hieble, 2011), so that we hypothesized that not only α_{1D}-adrenoceptors but also 5-HT$_{1A}$ 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-HT$_{1A}$ receptors (Dizeyi et al., 2004).

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Moreover, 5-HT$_{1A}$ receptor activation appears to increase prostate cell proliferation, via stimulation of the Akt/MAPK pathway (Hsiung et al., 2005; Dizeyi et al., 2011), and the 5-HT$_{1A}$ receptor antagonist NAN190 reduces prostate cell proliferation (Dizeyi et al., 2004). Based on these data, 5-HT$_{1A}$ receptors are considered as an attractive target for drug development in such context (Fiorino et al., 2014).

Previously, we showed that the *N*1-(2-methoxyphenyl)-*N*4-piperazine moiety confers affinity for α_{1A}-, α_{1D}-adrenoceptors and 5-HT$_{1A}$ receptors (Chagas-Silva et al., 2014). Here, we investigate the *in vitro* and *in vivo* pharmacological characteristics of three *N*-phenylpiperazine derivatives LDT3, LDT5 and LDT8 (European patent office, application No. 13733873.7-1451; USPTO application No. 14370646). Our results show that LDT3 and LDT5 are very potent multi-target antagonists of both α_{1A}- and α_{1D}-adrenoceptors, and also of 5-HT$_{1A}$ receptors. Also, these compounds inhibit the increase of rat intraurethral pressure (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.

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 UFRJ, 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 (UFRJ).

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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 (CEUA), under the license DFBC-ICB-011 (2008). Animals were kept under a 12/12 h 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, United States). 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, Brazil), and were available in the monohydrochloride form, as previously described for other *N*-phenylpiperazine derivatives (Romeiro et al., 2011). IR-FT spectra (Supplemental Figure 1) were recorded on a Spectrum BX spectrometer (Perkin Elmer, Waltham, USA), ^{1}H-NMR (300 and 500 MHz, $CDCl_3$) (Supplemental Figure 2), and ^{13}C-NMR (75 and 125 MHz, $CDCl_3$) 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.

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Drugs and radioligands

Prazosin hydrochloride, pargyline hydrochloride, 5-hydroxytryptamine hydrochloride (5-HT), acetylcholine chloride, (R)-(-)-phenylephrine hydrochloride, L-adrenaline (+)bitartrate, (±)-propranolol hydrochloride, 4-fluoro-N-(2-[4-(2-methoxyphenyl)1-piperazinyl]ethyl)-N-(2-pyridinyl)benzamide dihydrochloride (*p*-MPPF), 8-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride (BMY7378), 8-hydroxy-2-(dipropylamino)tetralin hydrobromide (8-OH-DPAT), 2-methoxy idazoxan (RX821002), 3-quinuclidinyl benzilate (QNB), tamsulosin hydrochloride, ketanserin tartrate, polyethyleneimine, atropine sulphate and guanosine-5'-triphosphate (GTP) were purchased from Sigma-Aldrich (St. Louis, USA). [^{3}H]-prazosin (85 Ci/mmol), [^{3}H]-ketanserin (60 Ci/mmol), [^{3}H]-8-OH-DPAT (187 Ci/mmol) and [^{3}H]*p*-MPPF (74.2 Ci/mmol) were obtained from PerkinElmer (Waltham, USA). [^{3}H]RX821002 (60 Ci/mmol) and [^{3}H]-QNB (250 Ci/mmol) were obtained from Amersham (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 physiological solution ([prostate, mM]: NaCl 138, KCl 5.7, $CaCl_2$ 1.8, NaH_2PO_4 0.36, $NaHCO_3$ 15 and glucose 5.5; [aorta, mM]: NaCl 122, KCl 5, $NaHCO_3$ 15, glucose 11.5, $MgCl_2$ 1.25, $CaCl_2$ 1.25 and KH_2PO_4 1.25) (95% O_2 and 5% CO_2, 37°C). Prostate and denuded aorta segments were preloaded (60 min) with 10 or 20 mN, respectively, and washed twice. Tissues were contracted with 1 μM phenylephrine (aorta) or 60 mM KCl depolarizing solution (prostate). After a 60-min recovery period,

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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 min with the test compounds (10 or 50 nM), BMY7378 or tamsulosin (10 nM). The developed force was recorded using an FT-03 grass force transducer (Warwick, RI, USA) 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 California USA), 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 (EC_{50}) was estimated before and after treatment with the antagonists. An EC_{50} ratio (CR) was calculated for each drug concentration by dividing the EC_{50} value after treatment by the control value (before treatment). Drug affinity (K_B) was estimated using the Schild equation: log (CR-1) = log [B] - log K_B (Kenakin, 1993), where B is the antagonist.

Intracellular Ca^{2+} measurement

The effect of LDTs on human α_1-adrenoceptor subtypes was determined by measuring intracellular Ca^{2+} in rat-1 fibroblasts stably expressing α_{1A}-, α_{1B}- or α_{1D}-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 analogue G418 sulfate, 100 μg/ml streptomycin, 100 units/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 min at 37ºC, in 20 mM HEPES (pH 7.4) containing 120 mM NaCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.75 mM KCl,

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10 mM glucose, 1.2 mM $CaCl_2$, 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 sec, 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-sec intervals, using an Aminco-Bowman Series 2 luminescence spectrometer (Rochester, NY, USA). Peak fluorescence values were used for data analysis, and the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) was calculated as described previously (Grynkiewicz et al., 1985). Data were analyzed by computerized nonlinear regression of untransformed data (GraphPad Prism 5.0, GraphPad Software, San Diego California USA), to estimate the half-maximum inhibitory concentration (IC_{50}) 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 intraurethral pressure (in mmHg) were monitored continuously using a fluid filled pressure transducer (PowerLab, ADInstruments, 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

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(30 min), 0.1 µg.kg^{-1} of LDT3, 0.1 – 100 µg.kg^{-1} of LDT5, or 100 µl vehicle (saline) were injected i.v., *in bolus*. Data were analysed 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 intraurethral pressure (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 min, IUP was increased by an i.v. administration of 1-100 µg.kg^{-1} phenylephrine every 10 min. Alternatively, a single dose of 30 µg.kg^{-1} phenylephrine was injected 10 min 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 – 0.1 µg.kg^{-1} i.v.). Data were analyzed by nonlinear regression (GraphPad Prism 5.0, GraphPad Software, San Diego California USA) to determine the half-maximum effective dose (ED_{50}).

Binding assays with native receptors

Rat brains were removed to obtain the hippocampus (5-HT_{1A} receptor) and cortex (5-HT_{2A}, α_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

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rapid filtration under vacuum using glass fiber filters (GMF 3, Filtrak, Germany) pre-soaked in 0.5% polyethyleneimine ([^{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, Waltham, USA). 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 here 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 α_{1B} adrenoceptors. Nevertheless, the pK_d value of [^{3}H]-prazosin for α_{1B}-adrenoceptors was 9.29 (0.51 nM), which was close to the value previously reported (pK_d 9.98 ± 0.27; Ohmura and Muramatsu, 1995).

For 5-HT$_{1A}$ receptor assays, 50 µg membrane protein were incubated with LTDs (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 µM pargyline (for 15 min at 37°C) or 0.5 nM [^{3}H]-*p*-MPPF (antagonist) and 1 mM GTP (for 45 min at 37°C). Nonspecific binding was determined in the presence of 10 µM 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 ($K_{i\ High}$) and for antagonist ([^{3}H]-*p*-MPPF) binding in the presence of a high concentration of GTP ($K_{i\ Low}$). $K_{i\ Low}/K_{i\ High}$ values higher than 1.0 indicate agonism, values close to 1.0 suggest antagonism and values lower than 1.0 indicate inverse agonism (Noël et al., 2014).

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Rat livers (α_{1B}-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 5,000 x g_{max}, for 20 min at 4°C. Supernatants were ultracentrifuged at 100,000 x g_{max}, for 60 min 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 µg liver membrane protein were incubated with test compounds (10^{-9} – 10^{-6} M) and 0.1 nM [^{3}H]-prazosin, in binding buffer containing 1 mM EDTA, for 45 min at 30ºC. Nonspecific binding was defined in the presence of 1 µM prazosin. Alternatively, saturation assays were performed using prazosin at concentrations ranging from 0.0001 to 0.1 µM (Chagas-Silva et al., 2014).

The binding assays for the α_2-adrenoceptors, 5-HT_{2A} and muscarinic receptors are described in the supplementary material.

Analysis of binding assays

Data were analyzed by computerized nonlinear regression of untransformed data (GraphPad Prism 5.0, GraphPad Software, San Diego California USA), to estimate the half-maximum inhibitory concentration (IC_{50}) of test compounds or radioligand K_d values. Dissociation constants (K_i) were calculated using the Cheng and Prusoff equation (Cheng and Prusoff, 1973).

The K_d of [^{3}H]-prazosin for α_{1B}-adrenoceptors was 0.51 nM, the K_d of [^{3}H]RX821002 for α_{2A}-adrenoceptors was 2.05 nM and the K_d of [^{3}H]-*p*-MPPF for 5-HT_{1A} receptors was 0.86 nM (Chagas-Silva et al., 2014). The K_d values of [^{3}H]-8-OH-

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DPAT for 5-HT_{1A} receptors (0.7 nM) and of [^{3}H]-ketanserin for 5-HT_{2A} 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% CO_2), until confluence. For cell growth assays, 5 x 10^3 or 3 x 10^3cells/well (for DU145 and BPH, respectively) were seeded in 96-well plates and cultured in serum-free medium for 24h, and then incubated for 48h 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 24h). Cell growth was evaluated by counting of viable cells using Trypan blue as an exclusion dye or by the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Data were expressed as the percentage of the control condition (vehicle).

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), respectively. ANOVA was followed by *post hoc* Dunnett’s or Newman-Keuls test. The F values calculated with the software GraphPad Prism 5.0 for the ANOVA prior to post-hoc tests are indicated in the legend of the tables and figures. Differences were considered statistically significant if $P < 0.05$.

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Results

LDT3, LTD5 and LDT8 have high affinity for native α_{1A}- and α_{1D}-adrenoceptors, but not for off-target receptors.

In assays using rat prostate, where the subtype α_{1A}-adrenoceptor is the most important for contraction (Hiraoka et al., 1999), our results indicate that LDT3, LDT5 and LDT8 have high affinity for α_{1A}-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, where the main α_1-adrenoceptors responsible for contraction belong to the α_{1D}-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 α_{1D}-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 = 2.95 nM; Table 2).

Analysis of the monophasic binding competition curves obtained with LDTs indicates that LDTs have lower affinity for α_{1B}-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 α_{1D}- and α_{1A}-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 α_{1B}- than for the α_{1D}-adrenoceptors (Table 2). In this experimental condition,

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tamsulosin showed a K_i value of 5.9 nM for α_{1B}-adrenoceptors (Chagas-Silva et al., 2014), in agreement with previous reports (Williams et al., 1999; Pulito et al., 2000).

We also evaluated the affinity of LDT3, LDT5 and LDT8 for the BPH off-target α_2-adrenoceptors and muscarinic receptors, using binding competition assays. LDTs showed K_i values in the micromolar range (0.2 – 108 µM), indicating a very low affinity for α_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 sub-types 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 towards 5-HT$_{1A}$ receptors. For this purpose we used the K_i ratio method, a functional binding assay that we recently described and compared to two other methods (GTP-shift and [^{35}S]-GTPγS 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), while LDT8 had a K_i ratio value compatible with a partial agonist. Since antagonism at the 5-HT$_{1A}$ receptor is supposed to be a pre-requisite 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.

LDT3 and LDT5 inhibit the proliferation of prostate cells from BPH patients.

LDT3 and LDT5 behaved as high-affinity antagonists of rat α_{1D}-adrenoceptors and 5-HT_{1A} receptors (Table 2 and 3); thus, we decided to verify whether these compounds had anti-proliferative 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 α_{1D}-adrenoceptors and 5-HT_{1A}-receptor inhibition, respectively. The LDTs concentration used (50 nM) corresponded to ~30-50 fold the estimated *in vitro* affinity of these compounds for α_{1D}-adrenoceptors and 5-HT_{1A} receptors. Counts of viable cells grown for 48h 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 for BMY7378 (Fig. 2A) and *p*-MPPF (Fig. 2B). We did not observe clear cytotoxic effects when LDTs were used alone (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 *K*i we reported for the α_{1D}-adrenoceptors (Table 2; Chagas-Silva et al., 2014). Similar effects of LDTs on BPH cells were obtained using the MTT assay ($P < 0.001$; Supplemental Figure 3).

In agreement with the results of cell proliferation assays using non-transformed BPH patient cells (Fig. 2), LDT3 and LDT5 also inhibited the 5-HT-stimulated growth of the prostate cancer cell line DU-145 (Supplemental Figure 4). Interestingly, LDT8 stimulated DU-145 cell growth (Supplemental Figure 4), which is compatible with the partial 5-HT_{1A} agonist activity detected for this compound in binding assays (Table 3).

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LDT3 and LDT5 decrease phenylephrine-induced calcium elevation in cells overexpressing human α_{1A}- and α_{1D}-adrenoceptors.

To confirm the antagonistic properties of LDT3 and LDT5 towards different human α_1-adrenoceptor subtypes, we used a functional assay based on intracellular Ca^{2+} ($[Ca^{2+}]i$) elevation in Rat-1 cells overexpressing human α_{1A}-, α_{1D}- or α_{1B}-adrenoceptors (Vázquez-Prado et al., 1997). In Rat-1 cells expressing α_{1D}-adrenoceptors, stimulation with 100 µM phenylephrine (PHE) typically induced a pronounced and transient increase in $[Ca^{2+}]i$ levels (Fig. 3A). In contrast, we observed a considerably less pronounced increase in $[Ca^{2+}]i$ after phenylephrine stimulation in cells incubated with LDT3 (Fig. 3B and C) or LDT5. For these α_{1D}-adrenoceptors, the mean pIC_{50} (and SD) values of LDT3 and LDT5 were 8.5 ± 0.67 (n = 6) and 8.38 ± 0.06 (n = 4), respectively (Supplemental Figure 5). The pIC_{50} value of BMY7378 was 8.53 ± 0.08 (n = 3). LDT3 and LDT5 also had a high potency at α_{1A}-adrenoceptors, with pIC_{50} 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). On the other hand, LDT3 and LDT5 had considerably lower affinity for α_{1B}-adrenoceptors, with pIC_{50} values for inhibition of $[Ca^{2+}]i$ elevation of 6.10 ± 0.29 and 5.88 ± 0.34 (n = 3), respectively ($P < 0.05$ versus α_{1A}- and $P < 0.001$ versus α_{1D}-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 α_1-adrenoceptors subtypes was $\alpha_{1D} > \alpha_{1A} > \alpha_{1B}$ (LDT3: $F_{2,9} = 19.41$, $P = 0.0005$; LDT5: $F_{2,6} = 37.08$, $P = 0.0004$. $P < 0.01$ one way ANOVA followed by Newman-Keuls test).

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LDT3 and LDT5 prevent phenylephrine-induced increase in intraurethral pressure, and do not affect basal blood pressure.

As prostate contraction increases intraurethral pressure (Akiyama et al., 1999), we investigated the effect of LDTs on rat intraurethral pressure (IUP) *in vivo*. Phenylephrine increased IUP in a dose-dependent manner, with an ED_{50} value of 7.5 $\mu g.kg^{-1}$ (Fig. 4A). Pre-treatment with either LDT3 or LDT5 (0.1 $\mu g.kg^{-1}$, i.v.) prevented the phenylephrine-induced increase in IUP (30 $\mu g.kg^{-1}$), and LDT5 was more effective than LDT3 (Fig.4B). In higher doses both compounds fully blocked the phenylephrine effect. The mean ED_{50} values of LDT3 and LDT5 (Fig. 4C), 0.15 and 0.09 $\mu g.kg^{-1}$, respectively, were higher than the ED_{50} value of tamsulosin (0.007 $\mu g.kg^{-1}$).

Considering that hypotension is a classical adverse effect of α_1-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 $\mu g.kg^{-1}$ LDT3 and LDT5 (mean and SD: -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 $\mu g.kg^{-1}$, i.v. reduced significantly the basal blood pressure (Fig. 4D).

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Discussion

Randomized controlled clinical trials have shown that blockage of prostatic α_1-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, α_{1A}-adrenoceptor or uroselective α_1-adrenoceptor antagonists show better tolerability (Chapple, 2005; McVary et al., 2011; Oelke et al., 2013). Nevertheless, some patients may be unresponsive to α_{1A}-adrenoceptor blockade (Kaplan, 2006), which limits the efficacy of α_{1A}-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 α_1-adrenoceptor blockers as compared to monotherapy with α_1-adrenoceptor blockers is only clearly observed after long-term therapy, while 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). Here, 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 α_{1D}-adrenoceptors and 5-HT_{1A} receptors, in addition to α_{1A}-adrenoceptor antagonism, could be particularly interesting because both receptors stimulate prostate cell growth (Dizeyi et al., 2004; Kojima et al., 2009a), α_{1D}-adrenoceptors mRNA expression is

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increased in BPH (Kojima et al., 2009a) and non-prostatic α_{1D}-adrenoceptors may contribute to bladder overactivity (Malloy et al., 1998; Kurizaki et al., 2001; Michel, 2010).

The α_{1A}-adrenoceptor mediates human (Forray et al., 1994) and rat (Hiraoka et al., 1999) prostatic contraction (Michel and Vrydag, 2006). LDT3, LDT5 and LDT8 had high affinity for α_{1A}-adrenoceptors in functional assays (Table 2). The affinities of LDT5 and LDT8 for rat α_{1A}-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 α_{1A}- and α_{1D}-adrenoceptors, LDT3 and LDT5 also have low affinity for the off-target α_{1B} subtype, in both species. α_1-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), while alfuzosin may be suitable for sexually active patients (as silodosin has the highest risk of ejaculatory dysfunction) (Chapple, 2005; Kim et al., 2014). Since human vascular expression of α_{1B}-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 classical adverse effect of therapy with α_1-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 intraurethral pressure (Akiyama et al., 1999). Data from *in vivo* assays indicated clearly that LDT3 and LDT5 blocked the increase in rat intraurethral pressure

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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} iv) was around 20% less effective than LDT5 (*data not shown*).Therefore, our results suggest that LDT3 and LDT5 are capable to relax 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 CHO cells that the stimulation of transfected human α_{1D}-adrenoceptor increases cell growth and ERK 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 α_1-adrenoceptors induces rat prostatic hyperplasia involving transforming growth factor (TGF)-β signaling (Kim et al., 2009). Actually, TGF-β signaling has been considered as one of the mechanisms that contribute to human prostate enlargement (Descazeaud et al., 2011).

α_{1D}-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 17 times higher affinity for human α_{1D}- than for α_{1A}- and α_{1B}-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

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prostatic α_{1D}-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 Figure 3), and of DU-145 prostate cancer cells in a way qualitatively similar to BMY7378, which suggests the role of α_{1D}-adrenoceptors (Supplemental Figure 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 α_1-adrenoceptor antagonists with a quinazoline moiety also inhibit prostate cell growth *in vitro;* however, this effect is independent of α_1-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-HT_{1A} receptors stimulate the growth of some cell types such as fibroblasts (Abdel-Baset et al., 1992). Benign and malignant prostate tissues express 5-HT_{1A} receptors and mounting evidence suggests that these receptors stimulate prostate cell growth via Akt/MAPK pathway (Abdul et al., 1994; Dizeyi et al., 2004, 2011). Based on binding assays, LDT3 and LDT5 have high affinity for 5-HT_{1A} receptors (Table 3). Importantly, LDT3 and LDT5 showed higher affinity for 5-HT_{1A} receptors than LDT66 (Chagas-Silva et al., 2014) and naftopidil, another *N*-phenylpiperazine compound (K_i = 107 nM; Borbe et al., 1991).

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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-HT_{1A} receptor antagonist *p*-MPPF (Fig. 2, Supplemental Figure 3), in agreement with the antiproliferative effect of another 5-HT_{1A} receptor antagonist (NAN 190) towards prostate cancer PC3 and DU-145 cell lines (Dizeyi et al., 2004). Moreover, while LDT3 and LDT5 blocked completely the agonist effect, LDT66 showed a partial inhibition (Chagas-Silva et al., 2014). On the other hand, LDT8 behaved as a partial agonist of 5-HT_{1A} 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 *N*1-(2-methoxyphenyl)-*N*4-piperazine scaffold confers affinity for α_1-adrenoceptors, as well as for 5-HT_{1A} receptors (Glennon et al., 1988; Leopoldo et al., 2004; Chagas-Silva et al., 2014), and unveil the multi-target antagonist behavior of the *N*-phenylpiperazine derivatives described here. In addition, LDT5 showed higher affinity for α_{1A}-, α_{1D}-adrenoceptors and 5-HT_{1A} receptors than the previous derivative LDT66, which has a hexil substitution in the N_4-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 here showed low affinity for off-target receptors, including α_{1B}- and α_2-adrenoceptors, as well as 5-HT_{2A} 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

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function (not shown), whose blockade can elicit potentially fatal cardiac arrhythmias (Priest et al., 2008) 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 α_1-adrenoceptors 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 α_{1A}-, α_{1D}-adrenoceptors and 5-HT_{1A} 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, 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

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Footnotes

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Legends for Figures

Figure 1. Effect of LDTs treatment on phenylephrine-induced contraction of rat prostate (A) and aorta (B), and on [^{3}H]-prazosin binding to rat liver membranes (C). These tissues are enriched in the α_1-adrenoceptor subtypes α_{1A} (prostate), α_{1D} (aorta) and α_{1B} (liver). During isometric contraction assays mediated by α_{1A}- and α_{1D}-adrenoceptors, tissues were pre-incubated with test compounds for 60 min before stimulation with phenylephrine. Note that the error bars showed here represent the SEM (instead of SD) of the means of 6-9 experiments using tissue samples from different animals. Competition binding data represent averaged curves from 3 to 5 independent experiments performed in triplicate.

Figure 2. Inhibition of α_{1D}-adrenoceptor- and 5-HT$_{1A}$ receptor-dependent prostate cell growth by LDTs. Trypan blue exclusion assays were performed using prostate cells from benign prostate hyperplasia (BPH) patients. BMY7378 (50 nM) and *p*-MPPF (50 nM, B) were used as selective antagonists of α_{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 x 10^5 cells. Data were expressed as mean and SD. n = 3-4 different cell cultures using cells from three donors. Assays performed in triplicates. (A) $F_{9,28} = 5.086$, $P = 0.0004$. (B) $F_{7,21} = 12.30$, $P < 0.0001$. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. the agonists (One-way analysis of variance (ANOVA) followed by the *post-hoc* Dunnett's test).

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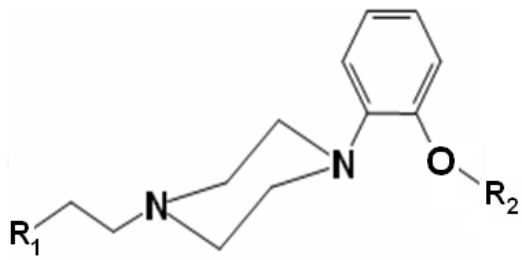
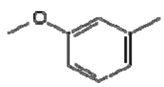
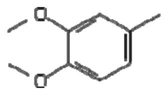
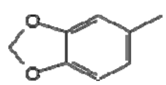
Figure 3. Effect of LDT3 treatment on phenylephrine (PHE)-induced intracellular calcium elevation in Rat-1 fibroblasts transfected with the human α_{1D}-adrenoceptor. The sharp increase in the intracellular calcium concentration ($[Ca^{2+}]i$) induced by 100 µM PHE (A; vehicle) was attenuated by treatment with 10 and 100 nM LDT3 (B and C, respectively).

Figure 4. Effects of LDT3 and LDT5 on intraurethral pressure modulation by phenylephrine. (A) Dose-response curve for the effect of phenylephrine (PHE, i.v.) on rat intraurethral pressure (IUP). The error bars showed here represent the SEM (instead of SD) of the mean (n=7). (B) Effect of PHE (30 µg.kg^{-1}) on IUP in the absence (white bar) or presence of pre-treatment with a single dose of LDT3 or LDT5 (0.1 µg.kg^{-1}, i.v.) (black bars). Data were expressed as mean and SD, n = 3-5. $F_{4,20} = 64.82$, $P < 0.0001$. ***$P < 0.001$ vs. PHE alone; ** $P < 0.01$ PHE after LDT3 vs. PHE after LDT5 (ANOVA followed by *post hoc* Newman-Keuls test). (C) Dose-response curves of LDT5 (n = 4) or tamsulosin (n = 3) on IUP. The error bars showed here represent the SEM (instead of SD) of the mean. (D) Effect of different doses of LDT5 on basal blood pressure. Data were expressed as mean and SD, n = 3-6. $F_{4,19} = 10.97$, $P < 0.0001$. ***$P < 0.001$ vs. vehicle (One-way analysis of variance (ANOVA) followed by the *post-hoc* Dunnett's test).

Tables

Table 1. Chemical structure of the *N*-phenylpiperazine derivatives LDT3, LDT5, LDT8 and LDT66.



LDT	R_1	R_2	Name
3		$-CH_3$	1-(2-methoxyphenyl)-4-[2-(3-methoxyphenyl)ethyl]piperazine
5		$-CH_3$	1-(2-methoxyphenyl)-4-[2-(3,4-dimethoxyphenyl)ethyl]piperazine
8		$-CH_2CH_3$	1-(1,3-benzodioxol-5-ylethyl)-4-ethoxyphenyl)piperazine
66*	$-(CH_2)_5CH_3$	-CH3	1-(2-methoxyphenyl)-4-hexylpiperazine

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

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Table 2. Affinity of *N*-phenylpiperazine derivatives for native rat α_{1D}- and α_{1A}-adrenoceptors.

receptor	compound	Control EC$_{50}$ (μM) [log EC$_{50}$ ± SD]	Treated EC$_{50}$ (μM) [log EC$_{50}$ ± SD]	log K_B ± SD (M)	K_B (nM)	n
α_{1A}	**LDT3**	0.24 [-6.62 ± 0.19]	1.2 [-5.92 ± 0.20]	- 8.58 ± 0.28***	**2.62**	7
	LDT5	0.14 [-6.86 ± 0.27]	7.9 [-5.10 ± 0.37]	- 9.74 ± 0.35	**0.18**	13
	LDT8	0.25 [-6.60 ± 0.24]	14.8 [-4.83 ± 0.27]	- 9.76 ± 0.32	**0.17**	10
	LDT66[a]	-	-		**3.4**[&]	-
	tamsulosin[a]	-	-	- 10.08 ± 0.04	**0.08**	3
α_{1D}	**LDT3**[b]	0.15 [-6.87 ± 0.23]	3.55 [-5.45 ± 0.24]	-8.71 ±0.10*	**1.95**	6
	LDT5[c]	0.08 [-7.12 ± 0.23]	1.34 [-5.87 ± 0.23]	-9.23 ± 0.08***	**0.59**	8
	LDT8[c]	0.07 [-7.14 ± 0.09]	4.58 [-5.34 ± 0.14]	-9.75 ± 0.16***	**0.18**	9
	LDT66[a]	-	-		**2.18**[&]	-
	BMY 7378[a]	-	-	-8.53 ± 0.13	**3.03**	5
	tamsulosin[a]	-	-	9.99 ± 0.1	**0.1**	3

[a] Data from Chagas-Silva et al., 2014 used for comparison (with permission). [&] Calculated from pA_2 values by Schild regression of mean curves (Chagas-Silva et al., 2014 used for comparison (with permission).

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For α_{1A}-adrenoceptors, the EC_{50} and K_B values were estimated using isomeric contraction assays of rat prostate stimulated with phenylephrine, in the absence or presence of 10 nM of the antagonist. Tamsulosin (10 nM) was used as control.
For α_{1D}-adrenoceptors, EC_{50} and K_B values were estimated using isomeric contraction assays of rat aorta stimulated with phenylephrine in the absence or presence of 50 nM ([b]), or 10 nM ([c]) of the antagonist. BMY 7378 (10 nM) was used as a selective antagonist of α_{1D}-adrenoceptors.
Log K_B values were calculated individually.
$F_{3,29} = 27.10$, $P < 0.0001$ for α_{1A}-adrenoceptors. *** $P < 0.001$ compared to tamsulosin (one way ANOVA followed by a *post-hoc* Dunnett's test)
$F_{3,24} = 169.7$, $P < 0.0001$ for α_{1D}-adrenoceptor. * $P < 0.05$, $^{***}P < 0.001$ compared to BMY 7378 (one way ANOVA followed by a *post-hoc* Dunnett's test).

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Table 3. Affinity of LDT3, LDT5 and LDT8 for rat 5-HT_{1A} receptors.

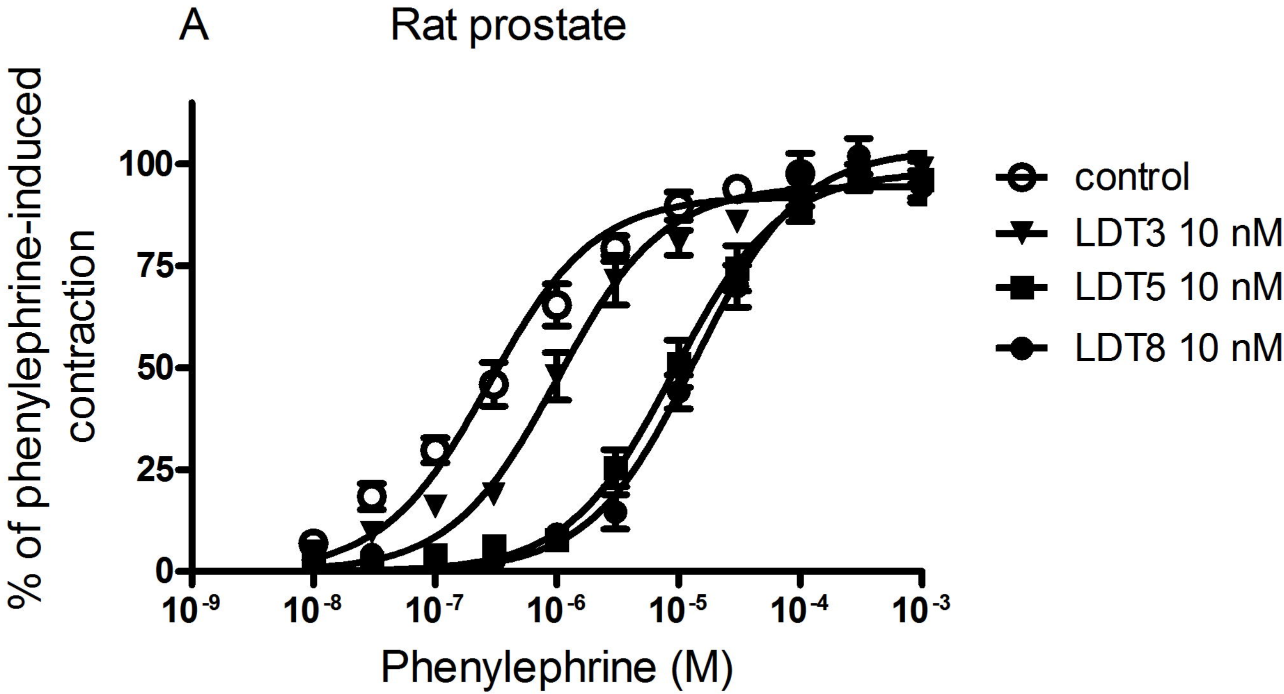
Compound	**$K_{i\ High}$, nM (n)** [pK_i ± SD]	**$K_{i\ Low}$, nM (n)** [pK_i ± SD]	**$K_{i\ Low}/K_{i\ High}$** [95% C.I.]
LDT3	1.12 (4) [8.95 ± 0.07]***	1.73 (3) [8.76 ± 0.19]*	1.53 [1.02-2.81]
LDT5	2.51 (4) [8.60 ± 0.05]***	6.91 (3) [8.16 ± 0.13]***	2.75 [1.68-4.50]
LDT8	0.009 (2) [11.05 ± 0.03]	0.62 (3) [9.21 ± 0.07]	66.9 [45.0-99.8]
LDT66[a]	5.9 (4) [8.23 ± 0.3]***	10.2 (4) [7.99 ± 0.1]***	1.71 [0.32-9.20]
5-HT[a]	3.02 (3) [8.52 ± 0.03]	213 (6) [6.67± 0.15]	76.8 [40.5-146]

*K*i values were determined in competition binding assays with the agonist [^{3}H]8-OH-DPAT ($K_{i\ High}$) or antagonist [^{3}H]*p*-MPPF (in the presence of high GTP; $K_{i\ Low}$), using membrane preparations of rat hippocampus. pK_i values (*i.e.*, -log K_i) were expressed as arithmetic means and SD of (n) experiments. The $K_{i\ Low}/K_{i\ High}$ ratio is an estimate of the intrinsic activity towards 5-HT_{1A} receptors, where values significantly higher than 1 indicate agonist activity (5-HT, was used as an example of full agonist), while values close to 1 indicate antagonist activity. The 95% confidence intervals (C.I.) of the K_i ratios were calculated as previously described (Noël et al., 2014).

[a] Data from Chagas-Silva et al., 2014 with permission.

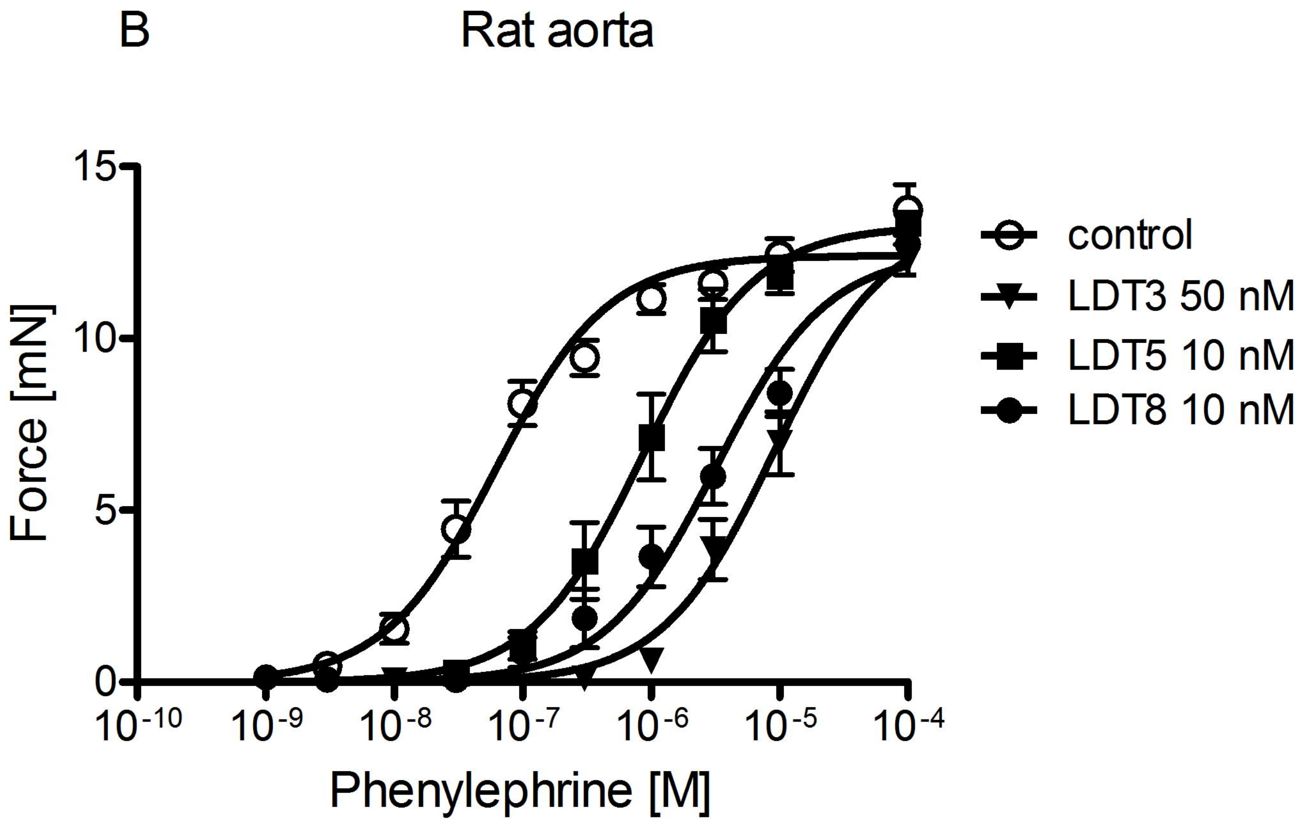
$F_{3,10}$ = 1471, $P < 0.0001$ for $K_{i\ high}$. *** $P < 0.001$ compared to LDT8 (one way ANOVA for LDTs followed by a *post-hoc* Dunnett's test).

$F_{3,9}$ = 55.97, $P < 0.0001$ for $K_{i\ low}$. * $P < 0.05$, *** $P < 0.001$ compared to LDT8 (one way ANOVA for LDTs followed by a *post-hoc* Dunnett's test).



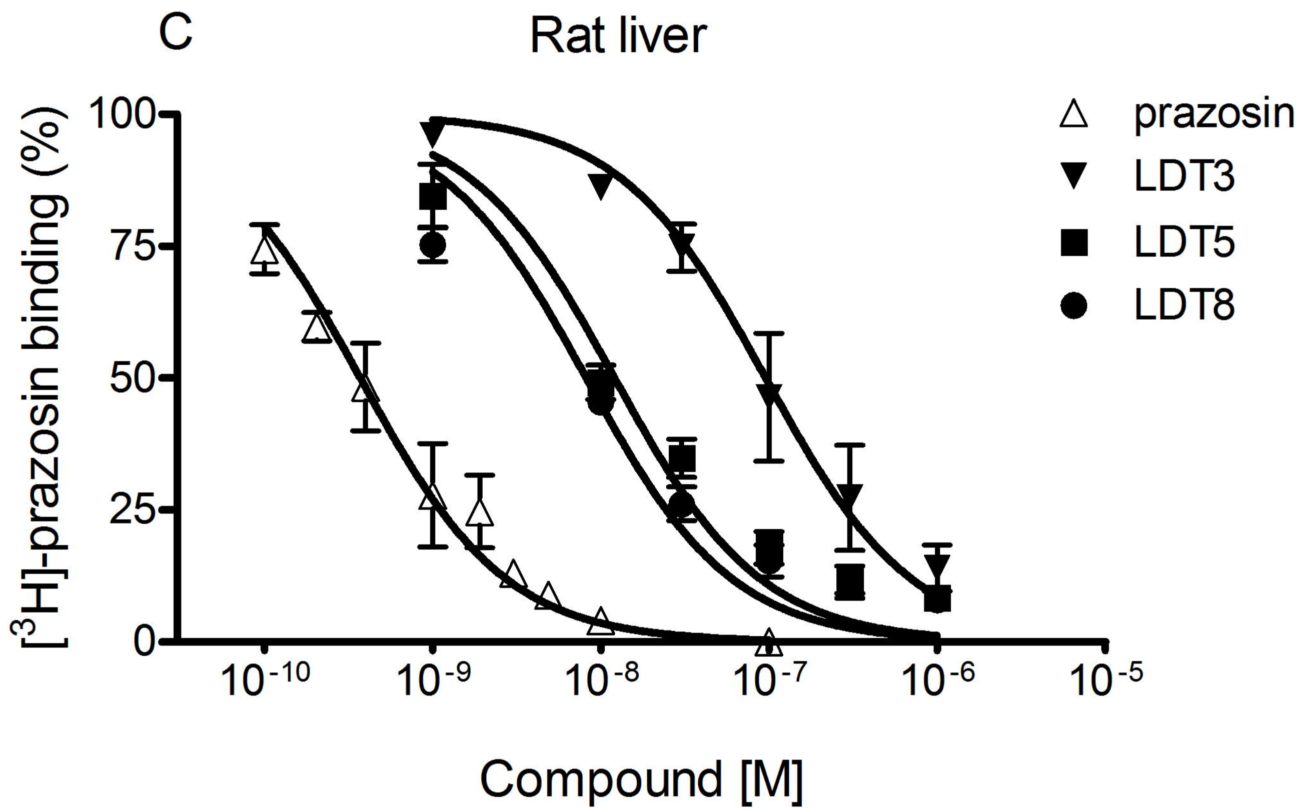
A
Rat prostate
% of phenylephrine-induced contraction
100
75
50
25
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Phenylephrine (M)
control
LDT3 10 nM
LDT5 10 nM
LDT8 10 nM

Fig 1A



B
Rat aorta
15
10
5
0
Force [mN]
10-10
10-9
10-8
10-7
10-6
10-5
10-4
Phenylephrine [M]
control
LDT3 50 nM
LDT5 10 nM
LDT8 10 nM

Fig. 1B



C
Rat liver
[3H]-prazosin binding (%)
100
75
50
25
0
10-10
10-9
10-8
10-7
10-6
10-5
Compound [M]
prazosin
LDT3
LDT5
LDT8

Fig. 1C

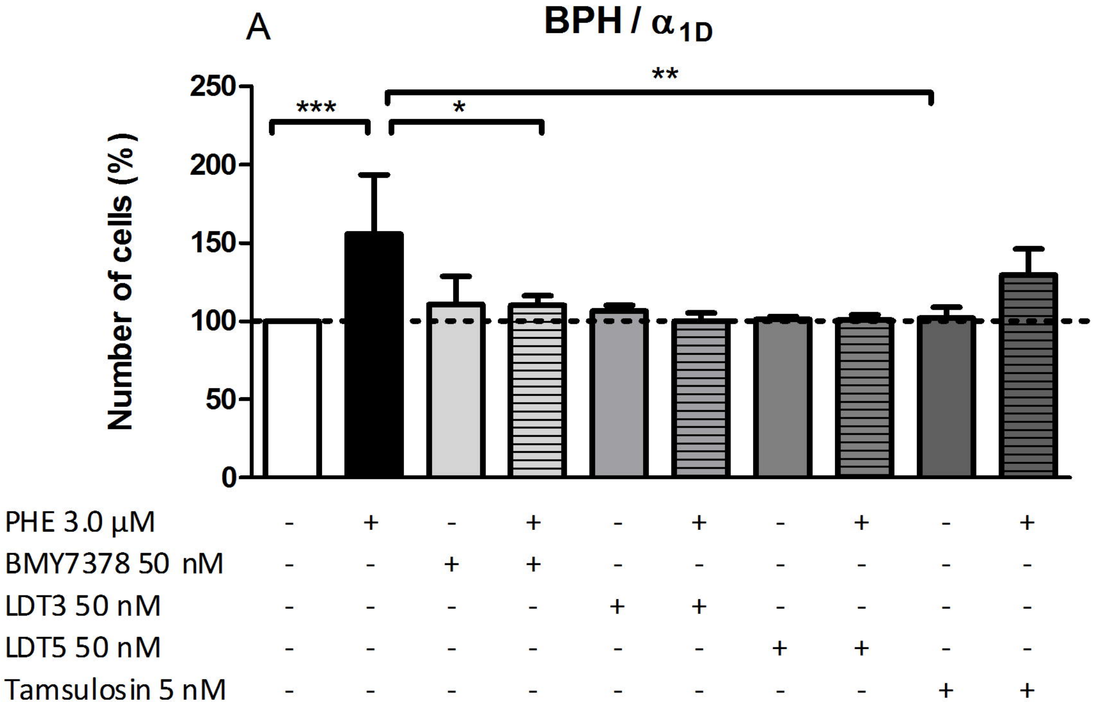

A
BPH / α1D
Number of cells (%)
250
200
150
100
50
0
***
*
**
PHE 3.0 µM - + - + - + - + - +
BMY7378 50 nM - - + + - - - - - -
LDT3 50 nM - - - - + + - - - -
LDT5 50 nM - - - - - - + + - -
Tamsulosin 5 nM - - - - - - - - + +

Fig. 2A

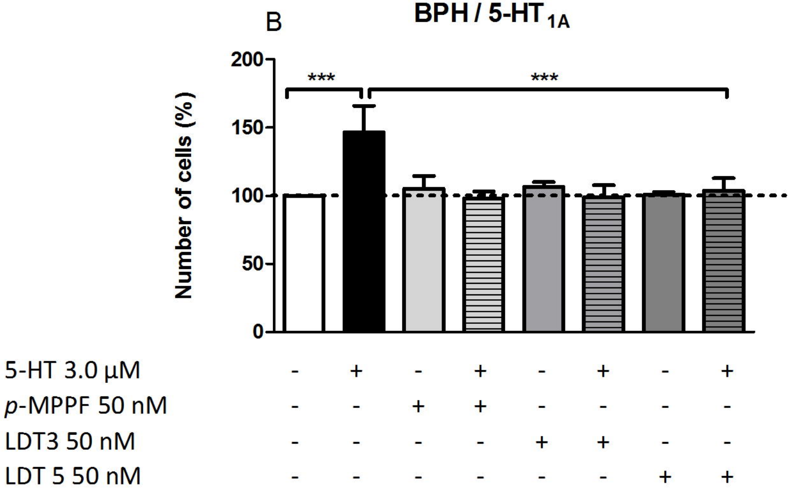

B
BPH / 5-HT 1A
Number of cells (%)
200
150
100
50
0
***
***
5-HT 3.0 µM - + - + - + - +
p-MPPF 50 nM - - + + - - - -
LDT3 50 nM - - - - + + - -
LDT 5 50 nM - - - - - - + +

Fig. 2B

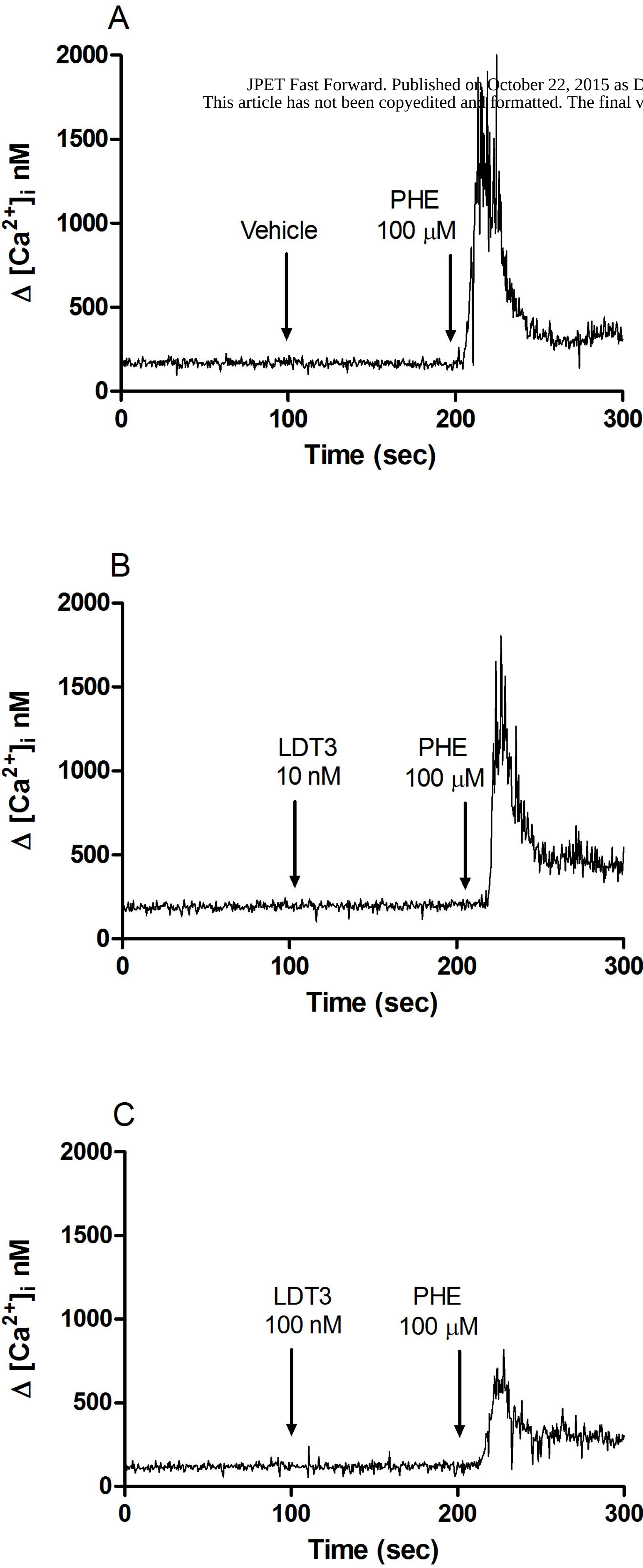

A
Vehicle
PHE 100 μM
B
LDT3 10 nM
PHE 100 μM
C
LDT3 100 nM
PHE 100 μM
Δ [Ca2+]i nM
Time (sec)
0
100
200
300
500
1000
1500
2000

Fig 3

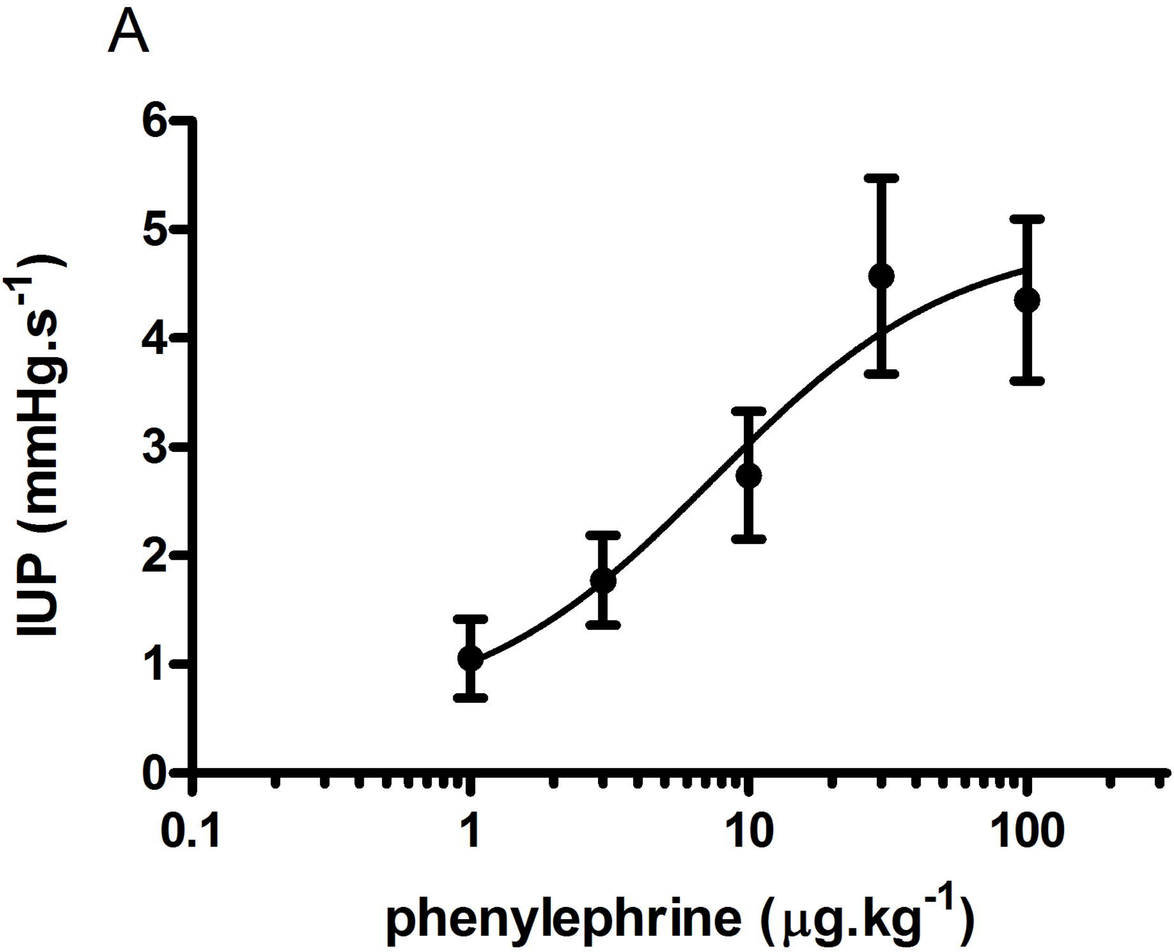
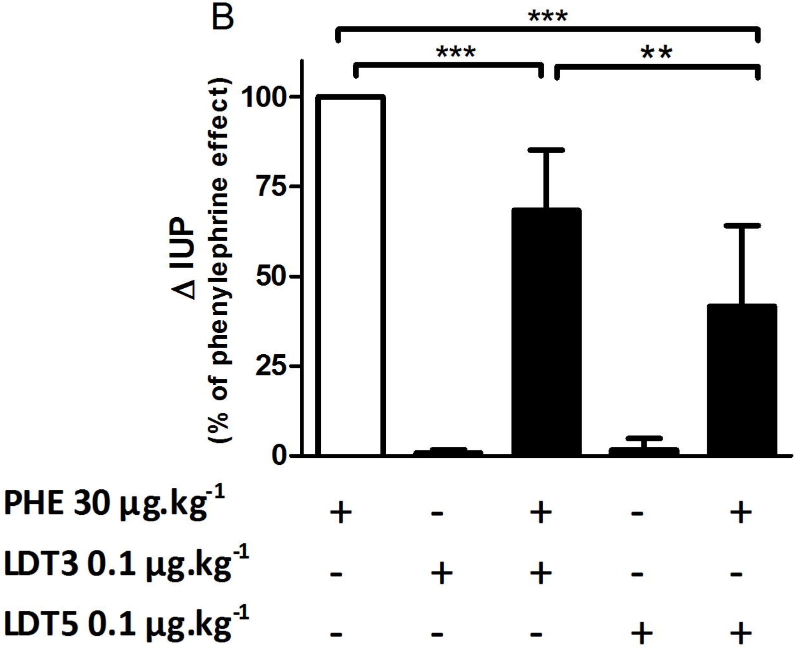


Fig. 4A



B
***
***
**
Δ IUP
(% of phenylephrine effect)
100
75
50
25
0
PHE 30 µg.kg-1
LDT3 0.1 µg.kg-1
LDT5 0.1 µg.kg-1

Fig. 4B

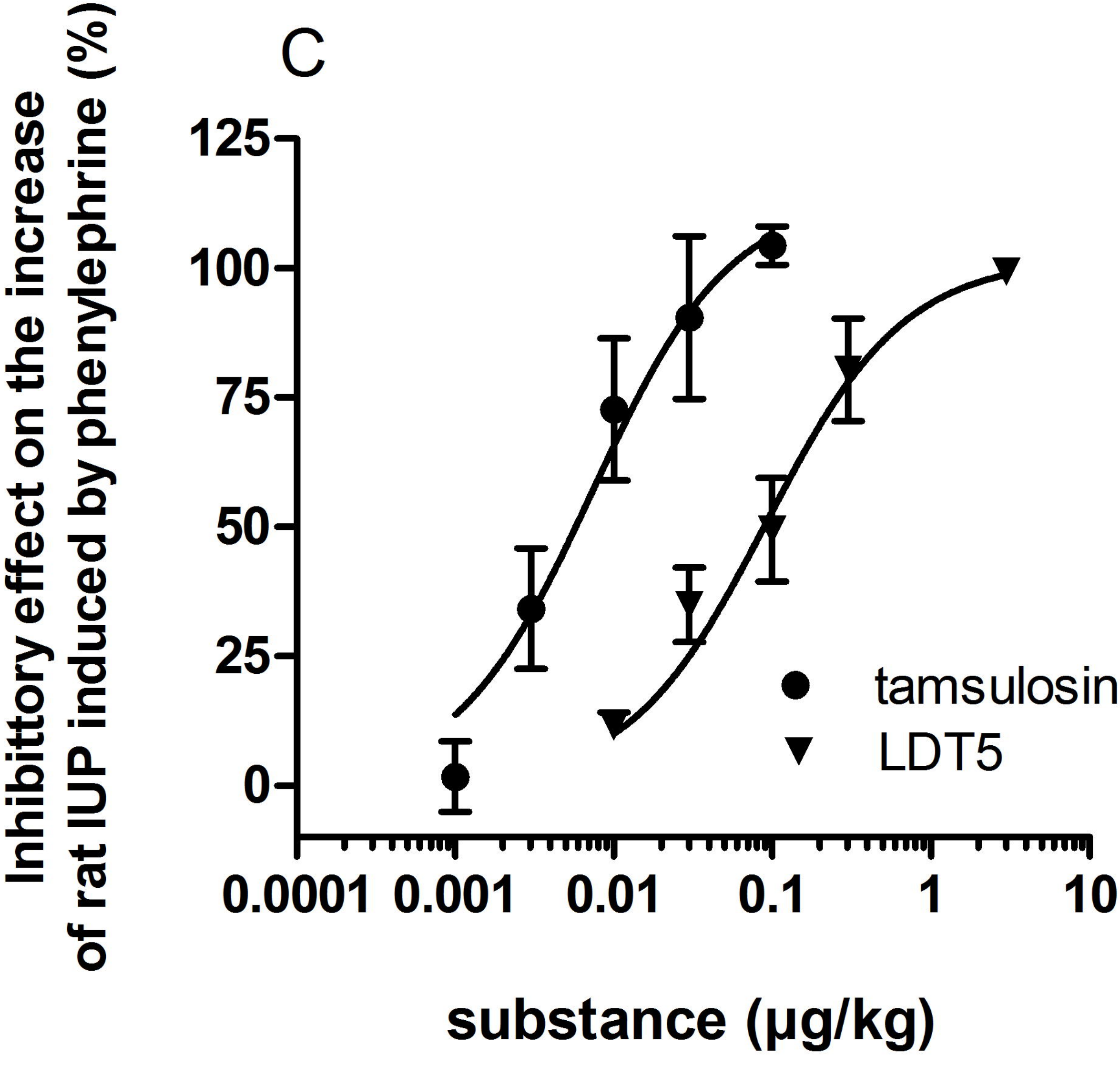

C
Inhibittory effect on the increase of rat IUP induced by phenylephrine (%)
125
100
75
50
25
0
0.0001
0.001
0.01
0.1
1
10
substance (µg/kg)
tamsulosin
LDT5

Fig. 4C

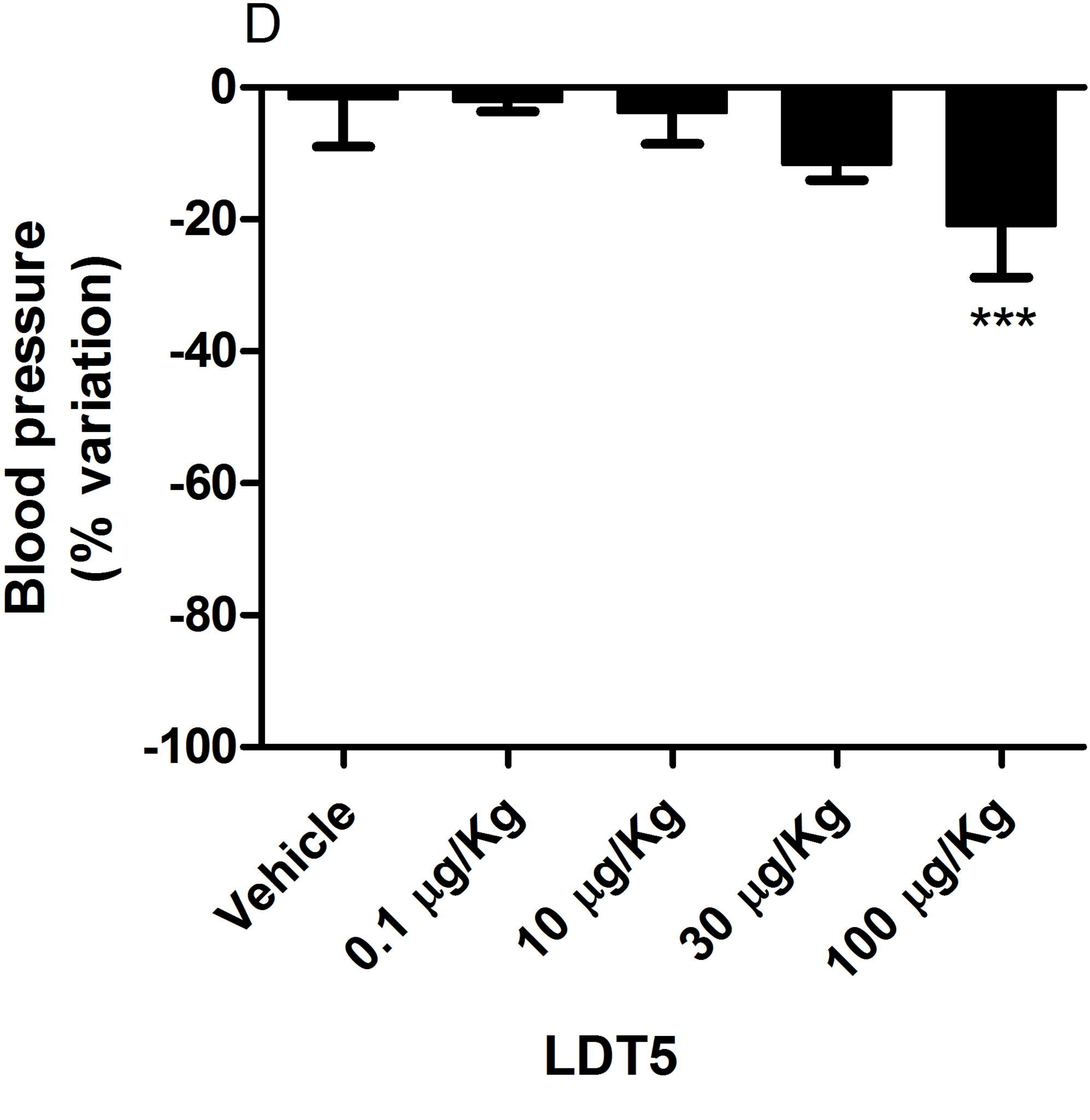

D
0
-20
-40
-60
-80
-100
Blood pressure
(% variation)
***
Vehicle
0.1 µg/Kg
10 µg/Kg
30 µg/Kg
100 µg/Kg
LDT5

Fig 4D