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TITLE PAGE

Revisiting the pharmacodynamics uroselectivity of Alpha1-Adrenergic Receptor Antagonists

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RUNNING TITLE PAGE

Running Title: Uroselectivity of alpha1-adrenergic antagonists

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Non-standard abbreviations:

AARA, α_1-AR antagonists; AR, α_1-adrenoceptor; BPH, benign prostatic hyperplasia; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl

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sulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LUTS, lower urinary tract symptoms; PBS, phosphate-buffered saline.

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Abstract

α_1-adrenoceptor (AR) antagonists are widely used for the relief of urinary retention secondary to benign prostatic hyperplasia (BPH). While the five FDA-approved α_1-AR antagonists (terazosin, doxazosin, alfuzosin, tamsulosin and silodosin) share similar efficacy, they differ in tolerability with reports of ejaculatory dysfunction. The aim of the present work was to revisit their α_1-AR subtype selectivity as well as of LDT5, a compound previously described as a multi-target antagonist of α_{1A}-/α_{1D}-AR and 5-HT_{1A} receptors, and to estimate their affinity for D_2, D_3 and 5-HT_{1A} receptors, putatively involved in ejaculatory dysfunction. Competition binding assays were performed with native (D_2, 5-HT_{1A}) or transfected (human α_{1A}-, α_{1B}-, α_{1Dt}-AR and D_3) receptors for determination of drug´s affinities. Tamsulosin and silodosin have the highest affinities for α_{1A}-AR, but only silodosin is clearly a selective α_{1A}-AR antagonist with K_i ratios of 25.3 and 50.2, for the α_{1D}- and α_{1B}-AR, respectively. Tamsulosin, silodosin and LDT5, but not terazosin, doxazosin and alfuzosin, have high affinity for the 5-HT_{1A}R (K_i around 5-10 nM), behaving as antagonists. We conclude that the uroselectivity of tamsulosin is not explained by its too low selectivity for the α_{1A}- vs. α_{1B}-AR and that its affinity for D_2 and D_3 receptors is probably too low for explaining the ejaculatory dysfunction reported for this drug. Present data also support the design of “better-than-LDT5” new multi-target lead compounds with pharmacokinetics selectivity based on poor brain penetration and that could prevent hyperplastic cell proliferation and BPH progression.

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Significance Statement

Present work revisits the uroselectivity of the five FDA-approved α_1 adrenoceptor antagonists for the treatment of benign prostatic hyperplasia (BPH). Contrarily to what has been claimed by some, our results indicate that the uroselectivity of tamsulosin is probably not fully explained by its too weak selectivity for the α_{1A} vs. α_{1B} adrenoceptors. We also show that tamsulosin affinity for D_3 and 5-HT_{1A} receptors is probably too low for explaining the ejaculatory dysfunction reported for this drug. Based on our lead compound LDT5, present data support the search for a multi-target antagonist of α_{1A}-α_{1D} and 5-HT_{1A} receptors with poor brain penetration as an alternative for BPH treatment.

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1. Introduction

Benign prostatic hyperplasia (BPH) is an age-related disease affecting the quality of life of men mainly due to bladder outlet obstruction among other bothersome lower urinary tract symptoms (LUTS) such as urgency and nocturia (Berry et al., 1984). α_{1A}-Adrenoceptors (ARs) and α_{1D}-ARs mRNA have been described in normal and hyperplastic stromal human prostates, and the expression of α_{1A}-ARs is upregulated during BPH (Kojima et al., 2006; Roehrborn and Shwinn, 2004; Walden et al., 1999; Nasu et al., 1996; Faure et al., 1994; Price et al., 1993). The stromal α_{1A}-ARs have been considered important for human prostate contraction (Forray et al., 1994), and consequently, for the dynamic component of BPH, so that their blockade would explain the observed relief of the micturition difficulties observed with antagonists. On the other hand, cellular proliferation in the periurethral region is related to the static component of BPH and classically treated at advanced stages of the disease (larger prostates) with the association of α_1-AR antagonists (AARA) and 5-α-reductase inhibitors (Alawamlh et al., 2018). However, other receptors are now being considered putative targets for blocking cellular proliferation, such as the α_{1D}-ARs and 5-HT_{1A} receptors (Oelke et al., 2013; McVary et al., 2011). 5-HT_{1A} receptors are considered as an attractive target for antiproliferative drugs since 5-HT acts as a growth factor on several types of non-tumoral and tumoral cells (Fiorino et al., 2014). Earlier works already reported that neuroendocrine cells are present in normal and malignant prostate tissue releasing 5-HT (Abrahamsson et al., 1986) and that prostate cells, including those from BPH patients, express 5-HT_{1A} receptors (Dizeyi et al., 2004). Moreover, these authors showed that prostate cell

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proliferation was reduced by NAN190, a 5-HT_{1A} receptor antagonist (Dizeyi et al., 2004). Finally, we previously showed that LDT5 inhibited the in vitro growth of prostate cells from BPH patients, induced by 5-HT, similarly to that observed for p-MPPF, a classical 5-HT_{1A} receptor antagonist (Nascimento-Viana et al., 2016). Based on these data, we proposed that a multitarget antagonist towards the α_{1A}-AR, α_{1D}-AR and 5-HT_{1A} receptor, such as LDT5 (Nascimento-Viana, 2016), could be a rationale non-hormonal alternative in the search of new drugs for the pharmacotherapy of BPH.

Moderate to severe LUTS associated with BPH are mainly treated with AARAs. The five FDA-approved AARAs for BPH treatment have similar efficacies, but they differ in tolerability (Oelke et al., 2013; Michel, 2010; Schwinn and Roehrborn, 2008). The so-called uroselective drugs (tamsulosin, silodosin, and alfuzosin) are better tolerated and have a lower incidence of orthostatic hypotension than the first-generation drugs (terazosin and doxazosin) (Hennenberg et al., 2014; Michel 2010; Nickel, 2006). As commented by Korstanje et al. (2011), uroselectivity has been classically defined either in terms of α_1-AR subtype selectivity (pharmacological uroselectivity), preferential reduction of urethral pressure vs. blood pressure in animals (functional/physiological uroselectivity) or desired clinical effects on obstruction and LUTS vs. unwanted adverse effects (clinical uroselectivity). As differences exist between the K_i values and selectivities for the five FDA-approved AARA among laboratories (Table 1, supplementary material), claims such as tamsulosin´s selectivity for α_{1A}-AR should be carefully checked.

Here, we compared these five drugs exactly in the same experimental conditions with respect to their affinities for the three human α_1-AR subtypes,

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together with our LDT5 compound. Furthermore, we considered not only their selectivity towards the classical off-target α_{1B}-AR but also towards the D_3 and 5-HT_{1A} receptors, putatively responsible for sexual disorders such as abnormal ejaculation reported for silodosin and tamsulosin (La Torre et al., 2016; Lepor, 2012; Andersson Abdel-Hamid, 2011; Wolters and Hellstrom, 2006; Giuliano, 2006).

Differently than silodosin and contrarily to what has been claimed by some, our results indicate that the uroselectivity of tamsulosin is probably not fully explained by its too weak α_{1A}- vs. α_{1B}-AR selectivity. We also showed that tamsulosin affinity for D_3 and 5-HT_{1A} receptors is probably too low for explaining the ejaculatory dysfunction reported for this drug. Finally, we discuss how multitarget antagonists of α_{1A}-α_{1D} and 5-HT_{1A} receptors, such as LDT5, could be planned for avoiding safety problems at the central nervous system.

2. Material and methods

2.1 HEK-293 cells transfected with human α1-ADR

Human embryonic kidney (HEK-293; ATCC® CRL-1573™) transfected with human α_{1A}-AR, α_{1B}-AR and α_{1Dt}-AR (Pupo et al., 2003). As the recombinant full length human α_{1D}-AR is poorly expressed in recombinant systems, a truncated mutant in which the first 79 aminoacids were deleted (α_{1Dt}-AR) was used to increase the number of binding sites (Pupo et al., 2003; Nojimoto et al., 2010) The cells were cultured in DMEM medium (GIBCO®) containing 25 mM glucose, 44 mM sodium bicarbonate, 10 % fetal bovine serum from South America, 1 % pyruvate and 1 % penicillin (10,000 U/ml) / streptomycin (10,000 µg/ml) (Invitrogen, USA) and incubated (37 °C, 5 % CO_2) until confluence when they

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were washed with 1 ml phosphate buffered saline (PBS) and scrapped to obtain the homogenate. Subsequently, the homogenate was centrifuged at 30,000 x g for 20 min at 4 °C, the supernatant discarded and the pellet resuspended in approximately 10 mL of solution (25 mM HEPES, 150 mM NaCl, 3 mM PMSF and 1 mM protease inhibitors cocktail, pH 7.4). This material was homogenized with an ultraturrax apparatus (twice for 15 seconds at a speed of 9,500 rpm). The homogenate was then centrifuged at 30,000 x g for 20 minutes at 4°C, the supernatant was discarded, and the new pellet was resuspended in buffer containing 25 mM HEPES, 150 mM NaCl, pH 7.4 (Akinaga et al., 2013).

2.2 Binding experiments

2.2.1 Binding to the α_1-ARs

The membrane preparation of transfected HEK-293 cells (150 µg protein) was incubated for 45 minutes at 30 °C in 1 ml medium containing 0.05 nM [^{3}H]-prazosin, Tris–HCl 50 mM (pH 7.4) and 1 mM EDTA (Nascimento-Vianna et al., 2016). Nonspecific binding was defined in the presence of 1 µM prazosin. The incubation was terminated by filtration, washing and treatment of the filters as described previously (Nascimento-Vianna et al., 2016).

2.2.2 Binding to the 5-HT_{1A} receptor.

For binding assays to the 5-HT_{1A} receptors, hippocampus of adult male Wistar rats were homogenized and centrifuged as previously described (Noël et al., 2014). Binding to the low-affinity and high-affinity states of the receptor was performed as detailed previously together with the rationale for estimating the intrinsic efficacy of the ligands by using the K_i ratio (Noël et al., 2014). The

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protein was incubated at 37 °C under yellow light for either 45 min with 0.5 nM [^{3}H]-p-MPPF, Tris–HCl 50 mM (pH 7.4) and 1 mM GTP (Low-affinity state) or for 15 min in a solution containing 1 nM [^{3}H]-8-OH-DPAT, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 10 mM pargyline and Tris–HCl 50 mM (pH 7.4) (High-affinity state).

2.2.3 Binding to the D_2 and D_3 receptor

For binding assays to the D_2-like receptors, striatum of adult male Wistar rats was homogenized and centrifuged as previously described (Pompeu et al., 2013; protocol N0. DFBCICB021, Institutional Ethical Committee for Animal Care from the Federal University of Rio de Janeiro). For binding to the D_3 receptor, we used commercially available (Chemiscreen^{TM}, Millipore) crude membrane preparations of recombinant Chem-1 cells that have been transfected with the cDNA encoding the human D_3 receptor (accession number NM_000796). Membranes, compounds and radioligand (0.1 nM [^{3}H]-YM-09151-2) were incubated at 37 ºC for 60 min under yellow light in a solution containing 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1 mM EDTA and Tris-HCl 50 mM (pH 7.4) as previously described (Betti et al., 2017).

2.3 Statistical analysis

Data were analyzed by non-linear regression using GraphPad Prism 6.0 (GraphPad Software, Inc., CA, USA) using the classical equations for simple concentration-effect curves (saturation experiments) and competition binding assays to estimate affinity (K_d) of the radioligand and potency (median inhibitory concentrations, IC_{50}) of the unlabeled competitor ligands, respectively. The affinity of the unlabeled competitor ligands (K_i) was calculated using the IC_{50}

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values and the Cheng Prusoff equation (Cheng and Prusoff, 1973). K_i values were expressed as geometric means with their 95 % confidence interval.

2.4 Drugs

[^{3}H]-prazosin (85 Ci/mmol), [^{3}H]-8-OHDPAT (154.2 Ci/mmol), [^{3}H]-p-MPPF (74.2 Ci/mmol) and [^{3}H]-YM-09151-2 (81.1 Ci/mmol) were purchased from PerkinElmer (USA). Alfuzosin hydrochloride, doxazosin mesylate, tamsulosin hydrochloride, and terazosin hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO) and silodosin from ShangHai Biochempartner Co.,Ltd. (China). LDT5 hydrochloride was synthesized as previously described for other *N*-phenylpiperazine derivatives (Romeiro et al., 2011). Stock solutions (1 and 10 mM) were made in sterile deionised water (LDT5) or 100 % DMSO (Sigma-Aldrich, St. Louis, MO) and then diluted in water. At the final concentration used (no more than 0.1 %) DMSO had no effect in our assays.

3. Results

3.1 Determination of K_d for [^{3}H]-prazosin binding to the three human α_1-ARs.

We first characterized the binding of [^{3}H]-prazosin to the three subtypes of human α_1-AR by performing saturation experiments at equilibrium in order to determine the K_d values in our experimental conditions. The K_d values (nM) were 0.475, 0.354 and 0.577 for the α_{1A}-, $\alpha_{1B,}$ and α_{1Dt}-ARs, respectively (geometric means, n=2), and were similar to values described elsewhere with these cells (Nojimoto et al., 2010).

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3.2 Determination of K_i values and selectivity of test compounds for binding at the human α_1-ARs.

As illustrated in Figure 1, we performed full competition curves for the three human α_1-AR subtypes with our six compounds using the antagonist [^{3}H]-prazosin as the radioligand. Note that the potency sequence is somewhat different for these three subtypes as exemplified by silodosin, one of the most potent for inhibiting [^{3}H]-prazosin binding to the α_{1A}-AR (Fig.1A) but the less potent for the α_{1B}-AR (Fig. 1B) and α_{1B}-AR (Fig. 1C).

Tamsulosin and silodosin have the highest affinities for the α_{1A}-AR but differ mainly with respect to their selectivity profile: whereas tamsulosin affinity for α_{1A}-AR is only slightly higher than for α_{1D} and α_{1B}-AR, with K_i ratio of 2.92 and 5.1, respectively, silodosin is clearly a α_{1A}-AR selective ligand with K_i ratios of 25.3 and 50.2, for the α_{1D}- and α_{1B}-AR, respectively (Table 1 and Supplemental Figure 1).

With respect to the third uroselective drug, alfuzosin has an affinity 2-4 times lower for the α_{1A}-AR than for the other two subtypes, as also observed with terazosin. Alfuzosin and terazosin showed similar affinities for α_{1A}-AR and similar selectivity profiles (Table 1 and Supplemental Figure 1).

Doxazosin has a similar affinity for the three subtypes. Our compound LDT5 has the same affinity for the α_{1A}- and α_{1D}-ARs being around 2-3 times higher than that for α_{1B}-AR, showing a selectivity profile similar to tamsulosin (Table 1 and Supplemental Figure 1).

3.3 Determination of affinity and intrinsic efficacy of test compounds at the 5-HT_{1A} receptor

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In order to determine the affinity of the six compounds to the 5-HT_{1A} receptor, we used a binding assay with the antagonist radioligand [^{3}H]-pMPPF and rat hippocampal membranes, as previously described (Noël et al., 2014).

Table 2 shows that LDT5, tamsulosin, and silodosin have a high affinity for this receptor, with K_i values around 5-10 nM, whereas alfuzosin, terazosin and doxazosin have a much lower affinity, with K_i values higher than 1 µM. Tamsulosin had a K_i value close to the one reported previously by others (4.4 nM, Leonardi et al., 1997). Considering the affinity for the main target receptor of BPH involved in contraction (α_{1A}-AR) as a reference, table 2 indicates that LDT5 has the same affinity for the 5-HT_{1A} (K_i ratio equal to 1.56) and that silodosin affinity for 5-HT_{1A} is relevant (K_i ratio around 10). On the contrary, albeit tamsulosin binds to 5-HT_{1A} at nanomolar concentrations, its affinity is about 33 times lower than for the α_{1A}-AR. With K_i ratios much higher than 100, alfuzosin, terazosin and doxazosin are to be considered highly selective α_{1A}-AR ligands towards the 5-HT_{1A} receptor.

As not only affinity but also intrinsic efficacy is important for pharmacological effect, we then used a previously validated functional binding assay (Noël et al., 2014) for the three compounds with relevant affinity for the 5-HT_{1A} receptor. As described in figure 2 for silodosin and tamsulosin, competition curves were performed either using the antagonist radioligand [^{3}H]-pMPPF in the presence of GTP (low-affinity state of the receptor) or the agonist radioligand [^{3}H]-8-OH-DPAT in the presence of divalent cations that favor the high-affinity state of the receptor. In such assay, the intrinsic efficacy of a compound is estimated by its ratio of K_i´s measured when the receptor is in the low- to high-affinity state. With K_i ratios not different from 1, LDT5 and silodosin

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are to be considered as neutral antagonists, whereas tamsulosin harbored a K_i ratio of 3.9, significantly different from 1 (Table 2). As this ratio is much smaller than the one reported for the full agonist 5-HT (76.8, see Noël et al., 2014), tamsulosin is to be considered as a weak partial agonist of this receptor.

3.4 Determination of affinity of tamsulosin and LDT5 at D_2 and D_3 receptors.

Due to the putative role of D_2 and, mainly, D_3 receptors as off-targets for drugs used in BPH therapy, we determined the affinity of tamsulosin and LDT5 for human D_3 receptors and D_2-like receptors present in rat striatal preparations (mainly D_2 receptors, according to Booze and Wallace, 1995). Table 3 shows that both compounds have a higher affinity for the D_3 than for the D_2 receptors. The K_i ratios (D_3 vs. α_{1A}-AR) are around 44 and 8 for tamsulosin and LDT5, respectively.

4. Discussion

4.1 Pharmacological selectivity of the five FDA-approved α_{1A}-AR antagonists for BPH treatment.

The *in vitro* off-target receptor binding is a well established method of de-risking used in drug discovery programs (Bowes et al., 2012), and is also the basis for defining if the so-called uroselectivity of some α_1–AR antagonists used for BPH treatment is due to pharmacological selectivity or to other reasons (see introduction). Since α_{1B}–ARs are not involved in the pathophysiology of BPH, and are expressed in several tissues including blood vessels, they have been considered for a long time as off-target for α_1-AR antagonists used in BPH due to the idea that its blockade was responsible for cardiovascular adverse effects,

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mainly postural hypotension (Michel, 2010). This idea has now been challenged due to the controversial role of the α_{1B}-AR in controlling blood pressure and to the good cardiovascular tolerability of alfuzosin, a non-selective antagonist (Akinaga et al., 2019; Michel, 2010). On the other hand, the α_{1A}–AR is considered the main target for BPH treatment due to the prominence of this sub-type in the human prostate and its role in prostate contraction (Forray et al., 1994). As a result of the classical view on BPH, the K_i ratio (α_{1B}-AR/α_{1A}-AR) was used for quantifying pharmacological selectivity of such drugs. As indicated in Table 4, our results support the claimed α_{1A} selectivity of silodosin, since the referred K_i ratio is about 50. On the other hand, our data do not support the usually claim that tamsulosin is a α_{1A}-AR (or $\alpha_{1A/1D}$-AR) selective antagonist. Indeed, our K_i ratio was 5.1, similar to the low values already reported in two other works (Table 4). Note that even the higher K_i ratios reported by two other groups (around 12) are not sufficient to support the claim, as also criticized by Leport et al. (2012) who considered that no clinical advantage could be attributed to a receptor selectivity of only about 10 times. Based on a 94-99% binding to plasma proteins (Flomax CR product monograph), we can estimate that the free maximal plasma concentration of tamsulosin at steady-state after daily administration of a controlled-release tablet containing 0.4 mg tamsulosin hydrochloride is below or around the K_i we measured for tamsulosin binding to the α_{1B} AR. As a consequence, a higher than 5 K_i ratio (α_{1B} vs. α_{1A}) would have a clinical relevance since the active (free) plasma concentration would be in the “selective” range. As an alternative, the explanation elegantly proposed by Sato et al. (2012) sounds plausible. These authors reported that the residence time of tamsulosin at the α_{1A}–AR was much higher than that at the α_{1B}-AR subtype,

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contrarily to what occurred with prazosin. Note that a pharmacodynamics selectivity is expected for drugs with a higher residence time at the target than at the off-target (Copeland et al., 2006). As an alternative hypothesis to explain the tamsulosin's reported uroselectivity, Korstanje et al. (2011) concluded that tamsulosin would exhibit a greater uptake into human prostate than would be expected from plasma concentrations based on differences in unbound drug fraction in human prostate (59%) and plasma (0.4%). Based on these data, the AUC (0,24 h) of unbound tamsulosin in prostate tissue was estimated to be 63-fold higher than the AUC (0,24 h) in plasma. As it is assumed that under equilibrium conditions diffusion of unbound drug will lead to equal drug concentrations in these two compartments, we cannot discard an experimental artifact since the unbound concentrations were not measured directly through in situ microdialysis, the gold standard approach for such experiments. Our data also confirm that the uroselectivity of alfuzosin is not due to a pharmacological selectivity between the α_1-AR subtypes, since its affinity for the α_{1A}-AR is even slightly lower (higher K_i ratios) than for the two other subtypes, as also observed for the two “old” non-selective α_1-AR antagonists terazosin and doxazosin (Table 4).

Besides the α_{1B}-AR, we also considered here the affinity of the five FDA-approved drugs towards the 5-HT_{1A} and D_2/D_3 receptors, which are poorly discussed in the literature for these drugs. Our interest was based on the proposal that these receptors participate in the central control of ejaculation and could be involved in the ejaculation disorders observed clinically in BPH patients, particularly those treated with silodosin or tamsulosin (La Torre et al., 2016; Leport, 2012; Wolters and Hellstrom, 2006; Giuliano, 2006). For silodosin,

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albeit harboring some relevant affinity for the 5-HT_{1A} receptor, the central effect could probably be discarded due to its apparently poor brain penetration (Okura et al., 2002). For tamsulosin, the situation is less clear, since some penetration into the brain has been reported albeit without quantitative data (Giuliano et al., 2006) whereas a low potential to cross the blood-brain barrier has also been reported by others (Soeishi et al., 1990, apud Andersson and Abdel-Hamid, 2011). Our data do not support the participation of the 5-HT_{1A}, D_2 and D_3 receptors in the ejaculation disorders due to a relatively low affinity of tamsulosin for these receptors. Note that the K_i value for the D_3 receptor reported by Kuo et al. (2000) was much lower than ours (0.28 nM vs. 15.7 nM) and that we did not find any apparent explication for such difference neither other data in the literature.

4.2 LDT5 and insight for putative new multi-target lead compounds.

Present data extend our previous data indicating that LDT5 could be considered a multi-target drug for the $\alpha_{1A/D}$–AR and 5-HT_{1A} receptors (Nascimento-Vianna et al., 2016). Previous estimates of affinity (K_B) for the α_{1A} and α_{1D}-AR were based on the antagonism of phenylephrine-induced isometric contractions of rat prostate and aorta, respectively, whereas affinity for the α_{1B} -AR was assessed by competition for [^{3}H]-prazosin binding to rat liver synaptosomes (K_i). Present affinity estimates were all obtained in binding experiments with membranes of cells transfected with each of the three human α_1–AR subtypes, a priori, a more suitable assay for a translational point of view. Albeit the affinity for the α_{1A}- and α_{1D} -ARs are lower (8-14 times), the ratio of *K*i´s for these two receptors is similar confirming that LDT5 is a high affinity $\alpha_{1A/D}$-AR ligand. Based on present

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data, the selectivity towards the off-target α_{1B}-AR should be lower than previously estimated (2.9 vs. 55 times). However, *in vivo* LDT5 showed an ED_{50} of 0.09 $\mu g.kg^{-1}$ for the reduction of intraurethral pressure and a similar dose (0.1 $\mu g.kg^{-1}$) did not cause any hypotensive effect (Nascimento-Viana et al., 2016), which could suggest a potential uroselective profile in rats.

Present data give support for designing “better-than-LDT5” new multi-target ($\alpha_{1A/D}$–AR and 5-HT_{1A} receptor) lead compounds. Indeed, as blockade of brain 5-HT_{1A} receptors could result in on-target adverse effect (see 4.1.), a pharmacokinetics selectivity based on poor brain penetration would be a strategy for such compounds, e.g. by designing a drug that would be a substrate of P-gp. This concern is also strengthened by the relatively high affinity we reported here for LDT5 binding to the D_3 receptor. Considering these data, LDT5 is no more considered as the ideal lead compound since the permeability assay with MDCK-MDR1 showed that it is not a substrate of P-gp (Noël et al., 2016). However, we suggest that the rationale of such multi-target drug for BPH treatment is maintained mainly based on our previous data with cells from BPH patients, since LDT5 inhibited prostate hyperplastic cell proliferation and reduced intraurethral pressure without hypotensive effects (Nascimento-Vianna et al., 2016).

Authorship Contributions

Participated in research design: Quaresma, Silva and Noël;

Conducted experiments: Quaresma, Pimenta and Santos;

Contributed with original material: Romeiro (LDT5 synthesis) and Pupo (α_1-AR transfected cells);

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Performed data analysis: Quaresma, Pimenta and Santos;

Wrote or contributed to the writing of the manuscript: Noël and Silva;

Revised the manuscript: Quaresma, Pupo and Romeiro

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Footnotes

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Legends for figures

Fig. 1. Effect of α_1-AR antagonists on the binding of [^{3}H]-prazosin to human α_{1A}-AR (A), α_{1B}-AR (B) and α_{1D}-AR (C). The membrane preparation of transfected HEK-293 cells (150 µg protein) was incubated for 45 minutes at 30°C in 1 ml medium containing 0.05 nM [^{3}H]-prazosin, Tris–HCl 50 mM (pH 7.4), 1 mM EDTA in the presence or absence of increasing concentrations of the tested compounds. The data represent the mean ± SEM of 3-4 independent experiments performed in triplicate.

Fig.2. Specific binding of [^{3}H]-8-OH-DPAT (square) and [^{3}H]-p-MPPF (circle) in rat hippocampal membranes in the presence of increasing concentrations of silodosin and tamsulosin. The protein was incubated at 37 °C for either 45 min with 0.5 nM [^{3}H]-p-MPPF, Tris–HCl 50 mM (pH 7.4) and 1 mM GTP (Low-affinity state) or for 15 min in a solution containing 1 nM [^{3}H]-8-OH-DPAT, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 10 mM pargyline and Tris–HCl 50 mM (pH 7.4) (High-affinity state). Data are expressed as means ± SEM of 3-5 individual experiments performed in triplicate.

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Table 1: Affinity (*K*i values) and selectivity (ratios of *K*i´s) for binding of α_1-AR antagonists to the three human α_1–AR subtypes.

Compounds	K_i (nM) (95 % CI) α_{1A} (1)	K_i (nM) (95 % CI) α_{1Dt} (2)	K_i (nM) (95 % CI) α_{1B} (3)	K_i Ratio (2)/(1)	K_i Ratio (3)/(1)
LDT5	3.82* [3] (1.57-9.29)	4.94* [3] (19.0-9.24)	9.86 [3] (6.77-14.4)	1.29	2.90
Tamsulosin	0.36 [3] (0.11-1.17)	1.05* [3] (0.37-2.91)	1.85** [3] (1.36-5.50)	2.92	5.10
Silodosin	0.44 [3] (0.22-0.85)	11.1**** [3] (5.17-23.8)	22.1**** [3] (9.37-52.2)	25.2	50.2
Alfuzosin	11.4** [4] (4.31-30.4)	4.83 [4] (2.56-9.12)	2.35 [4] (1.18-4.68)	0.42	0.20
Terazosin	11.2* [4] (7.75-16.1)	6.67 [4] (2.61-17.0)	3.63 [4] (1.83-7.23)	0.59	0.32
Doxazosin	3.60 [4] (1.16-11.1	1.58 [4] (0.99-2.53)	2.16 [4] (1.40-3.32)	0.44	0.60

K_i values are expressed as geometric means of [n] individual experiments.

One-way ANOVA and post-hoc *Holm-Sidak* test on pK_i values: LDT5: * p<0.05 vs. AR-α_{1B}; Tamsulosin: * p<0.05; ** p<0.01 vs. AR-$\alpha_{1A;}$ Silodosin: **** p<0.0001 vs. AR-α_{1A}; Alfuzosin: ** p<0.01 vs. AR-$\alpha_{1B\ ;}$ Terazosin: * p<0.05 vs. AR-α_{1B}

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Table 2: Affinity (K_i values) for binding to the 5-HT_{1A} receptor (Low-affinity state) and selectivity for binding to the α_{1A}-AR vs. 5-HT_{1A} receptor.

Compounds	5-HT_{1A} – Low K_i (nM) (95 % CI) [n]	K_i ratio 5-HT_{1A} Low /α_{1A}-AR	K_i ratio 5-HT_{1A} (Low/High)
LDT5	5.96 [3] (2.64 – 13.5)	1.56	1.17
Tamsulosin	11.9 [5] (7.96 – 17.8)	33.0****	3.91$^{\#}$
Silodosin	4.23 [3] (1.21 – 14.8)	9.61**	1.20
Alfuzosin	2,130 [3] (380 – 11,900)	186***	-
Terazosin	19,890 [3] (2,970 – 133,270)	1,776****	-
Doxazosin	4,240 [3] (1,750 – 10,270)	1,178****	-

K_i values are expressed as geometric means of [n] individual values calculated from competition curves using the antagonist radioligand (low-affinity state, see methods). The ratio of these K_i values and the K_i values for the α_{1A}-AR is a measure of selectivity. Intrinsic activity at the 5-HT_{1A} receptor was estimated by the ratio of K_i´s for the low- and high-affinity state of the receptor (Noël et al., 2014).

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$^{\#}$p<0.01, unpaired Student´s *t* test on pK_i values (5-HT_{1A} Low vs. 5-HT_{1A} High); **p<0.01; ***p<0.001; ****p<0.0001, unpaired Student´s *t* test on pK_i values (5-HT_{1A} Low vs. α_{1A}).

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Table 3: Affinity of tamsulosin and LDT5 for human D_3 and rat striatum D_2–like receptors and selectivity for binding to the α_{1A}-AR vs. D_3 receptor. K_i values are expressed as geometric means of [n] individual experiments.

Compounds	K_i (nM) (95 % CI) D_3	K_i (nM) (95 % CI) D_2	K_i ratio D_3 / α_{1A}
Tamsulosin	15.7 [3] (3.7 – 37.5)	88.9 [4] (73.7 – 128)	43.6***
LDT5	30.7 [3] (14.7 – 47.5)	68.7 [5] (57.0 – 82.8)	8.04***

***p<0.001, *t* test (pK_i D_3 vs. pK_i α_{1A}).

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Table 4: Selectivity (ratios of K_i´s) for binding to α_1–AR subtypes: between target subtypes and between the main target (α_{1A}) and off-target (α_{1B}) subtypes. Comparison between present data and data from the literature.

Compounds	K_i ratio α_{1Dt} / α_{1A}							K_i ratio α_{1B} / α_{1A}						
	Our data	1	2	3	4	5	6	Our data	1	2	3	4	5	6
LDT5	1.29		-	-	-	-	-	2.90		-	-	-	-	-
Tamsulosin	2.92	2.5	3.5	1.00	0.80	-	0.26	5.10	10.0	11.7	12.6	6.3	-	3.30
Silodosin	25.2	56.4	25.6	-	-	-	19.8	50.2	167	25.6	-	-	-	23.2
Alfuzosin	0.42		0.17	0.10	0.32	0.63	-	0.20		0.15	0.13	1.00	0.47	-
Terazosin	0.59		0.52	0.10	-	0.50	0.15	0.32		0.49	0.10	-	0.28	0.05
Doxazosin	0.44		-	1.26	1.26	0.60	-	0.60		-	1.00	0.32	0.38	-

(1) Tatemichi et al., 2006; (2) Sato et al., 2012; (3): Richardson et al., 1997, (4) Kenny et al., 1996, (5) Forray et al., 1994 (present nomenclature), (6) Ishiguro et al.,2002

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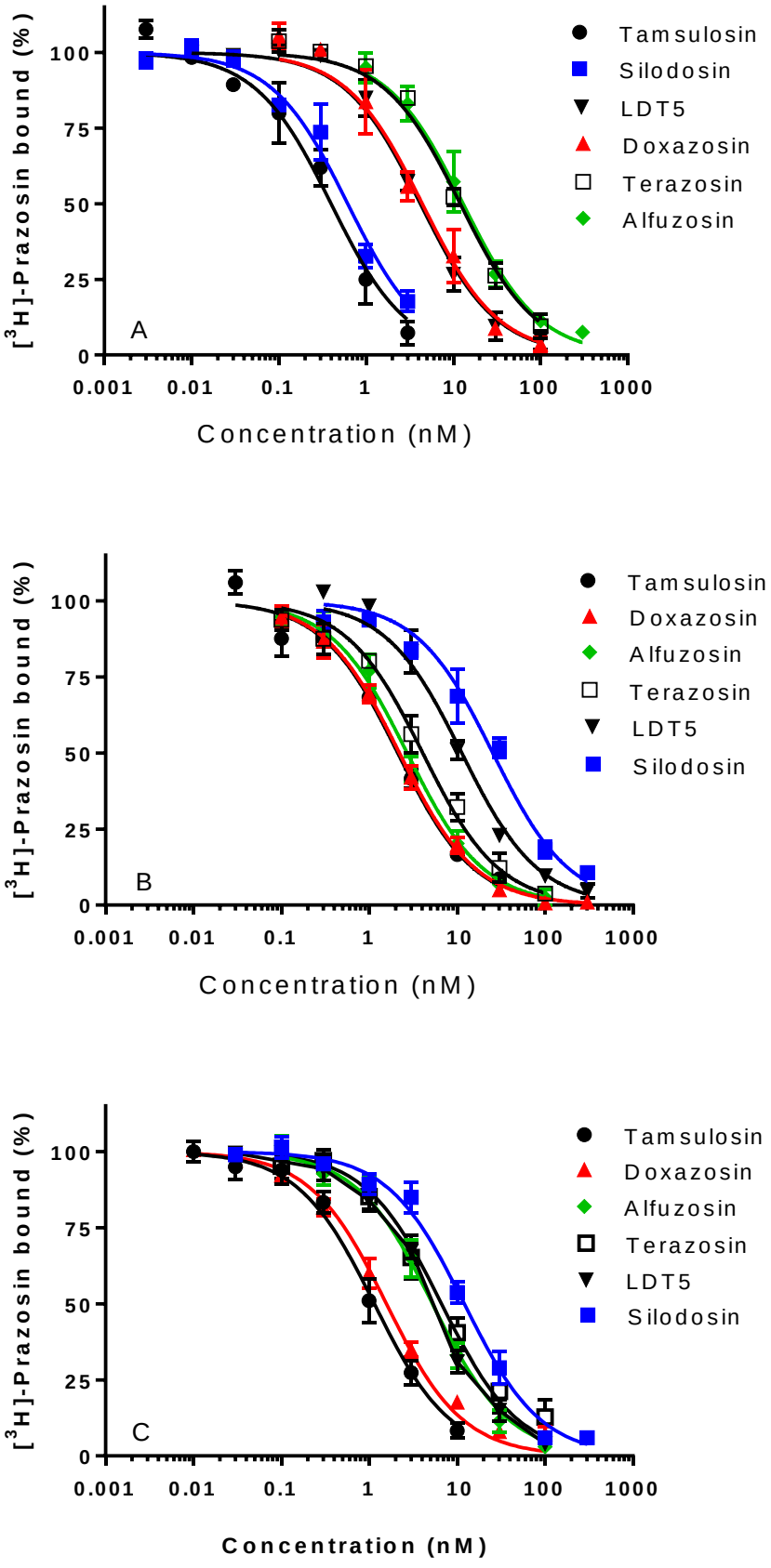
6 Figure 1



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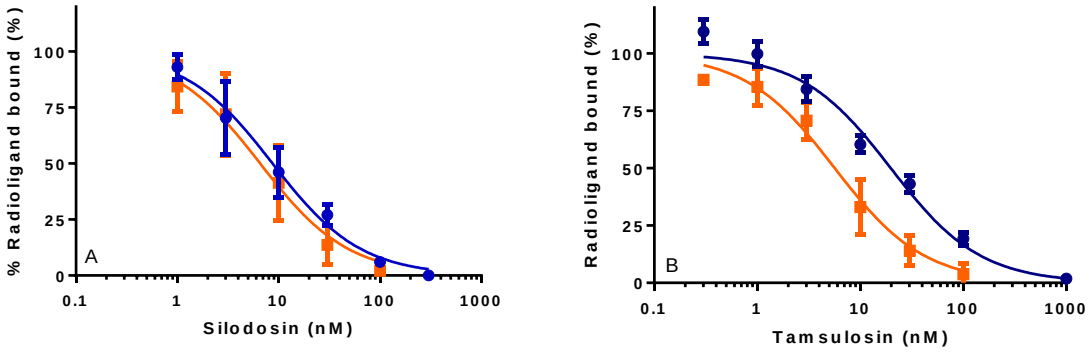
7 Figure 2



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