Supplemental Data

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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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 α_2-adrenoceptors, 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 LDTs (10^{-6} – 10^{-3}

M), at 25°C for 60 min. Atropine sulphate (10 μM) was used to determine non-specific binding (Chagas-Silva et al., 2014).

Statistical analysis

Otherwise indicated, data are expressed as means and SD. The significance of the differences among two or more conditions was determined by Student´s *t* test or one-way analysis of variance (ANOVA) followed by *post hoc* Dunnett's test, respectively.

Supplemental Table 1**.** Affinity of LDT derivatives for native rat α_2-adrenoceptors and muscarinic receptors.

	α_2-adrenoceptors		**muscarinic receptors**	
Compound (n)	**log IC_{50} ± SD (M)**	**K_i (µM)**	**log IC_{50} ± SD (M)**	**K_i (µM)**
LDT3 (4)	-5.97 ± 0.18	**0.93**	-4.26 ± 0.14	**56.7**
LDT5 (3)	-6.53 ± 0.09**	**0.24**	-3.98 ± 0.10*	**108**
LDT8 (4)	-6.22 ± 0.24	**0.55**	-4.48 ± 0.14	**34**
LDT66[a] (3)	-5.92 ± 0.13[b]	**0.81**	-3.80 ± 0.14	**52**
yohimbine (2)	-6.76 ± 0.07	**0.12**	---	---
pirenzepine (2)	---	---	-7.16 ± 0.04	**0.02**

IC_{50} values (expressed as mean ± SD) were calculated by nonlinear regression of data from binding competition assays using radiolabelled antagonists of α_2-adrenoceptors ([^{3}H]RX-821002) and muscarinic receptors ([^{3}H]-QNB). Yohimbine and pirenzepine were used as positive controls for α_2-adrenoceptor and muscarinic receptor antagonism, respectively. K_i values were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973), considering K_d values of 2.05 nM for [^{3}H]RX-821002 (Chagas-Silva et al., 2014) and 0.05 nM for [^{3}H]-QNB (Luthin and Wolfe, 1984). Experiments were performed in triplicates. [a] from Chagas-Silva et al., 2014 with permission; [b] n = 5.

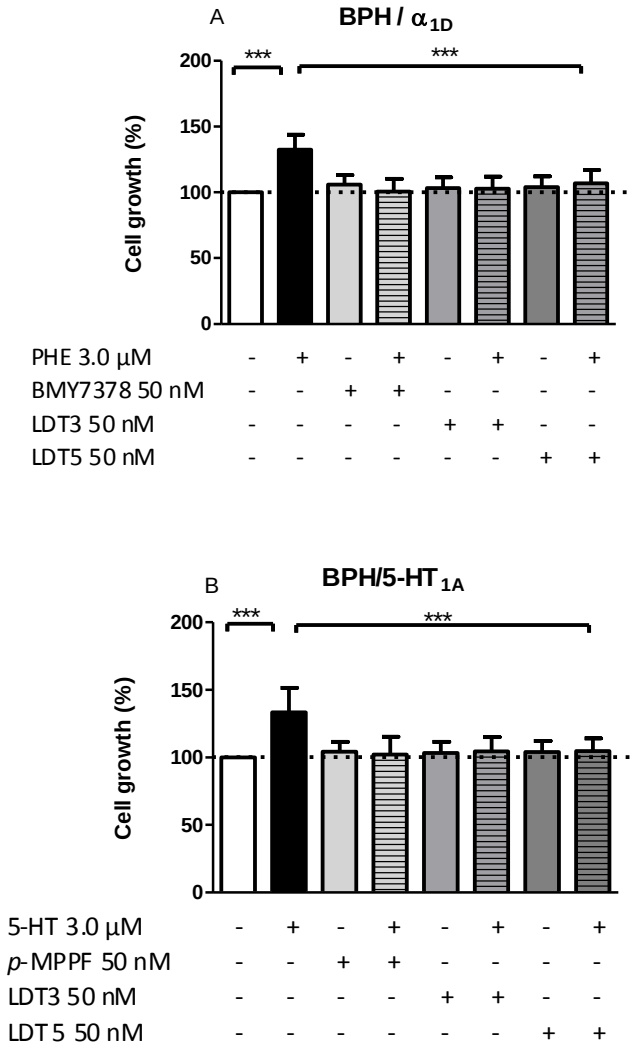
$F_{3,12}$ = 9.347, P = 0.0018 for α_2-adrenoceptors. ** P < 0.01vs. LDT3 (one way ANOVA followed by *post hoc* Dunnett's test)

$F_{3,10}$ = 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	5-HT$_{1A}$ K_i (M) (n) [log IC$_{50}$ ± SD (M)]	5-HT$_{2A}$ K_i (M) (n) [log IC$_{50}$ ± SD (M)]	Selectivity for 5-HT$_{1A}$ (5-HT$_{2A}$/5-HT$_{1A}$ K_i ratio)
LDT3	**1.12 x 10^{-9}** (4) [-8.56 ± 0.07] ***	**7.08 x 10^{-8}** (3) [-7.15 ± 0.38] #	63
LDT5	**2.51 x 10^{-9}** (4) [-8.21 ± 0.05] ***	**3.89 x 10^{-7}** (3) [-6.41 ± 0.03] #	155
LDT8	**8.85 x 10^{-12}** (2) [-10.66 ± 0.03]	**3.89 x 10^{-7}** (3) [-6.41 ± 1.21] **	43,949
LDT66[a]	**5.9 x 10^{-9}** (4) [-7.93 ± 0.40]***	**1.78 x 10^{-6}** (3) [-5.57 ± 0.28]#	300

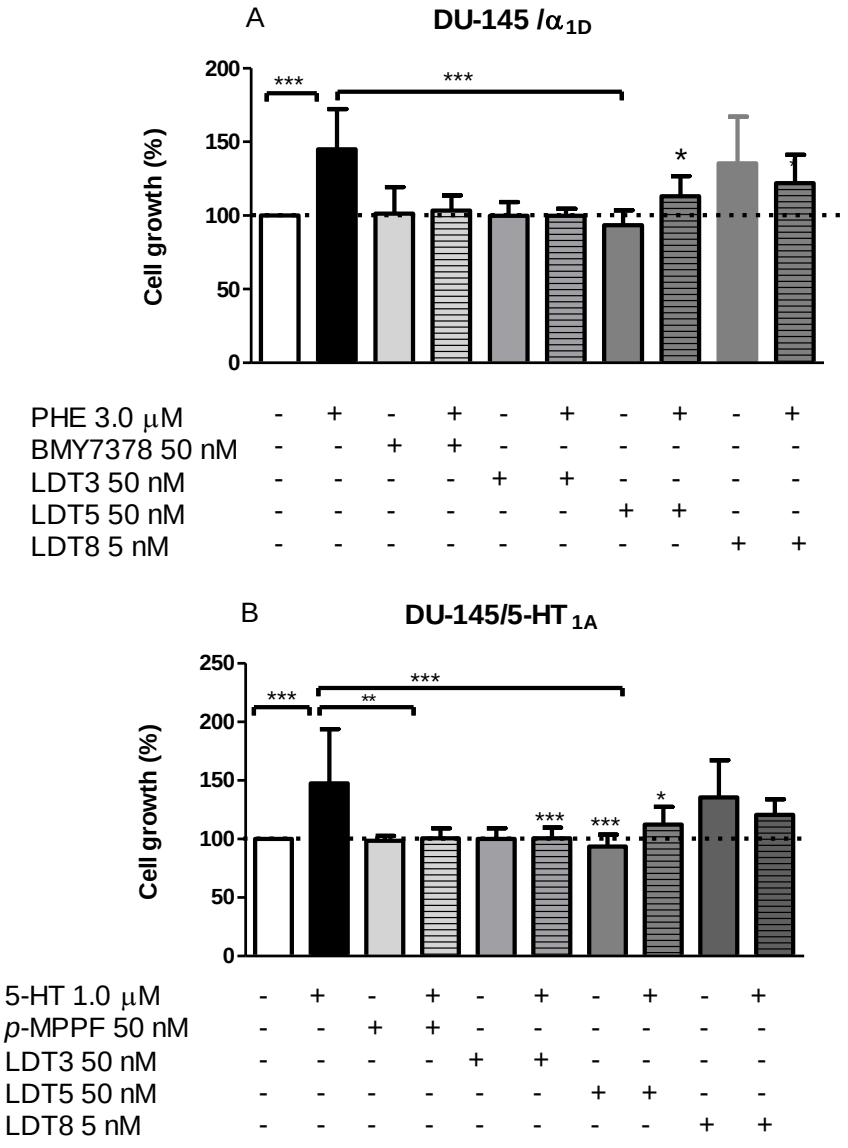
Data were obtained using binding competition assays with the radioligands [^{3}H]-8-OH-DPAT (5-HT$_{1A}$ receptor) and [^{3}H]-ketanserin (5-HT$_{2A}$ receptor). K_i values were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Experiments were performed in triplicates. [a] from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



Supplemental Figure 3. Inhibition of human hyperplastic prostate cell growth by LDT3 and LDT5.

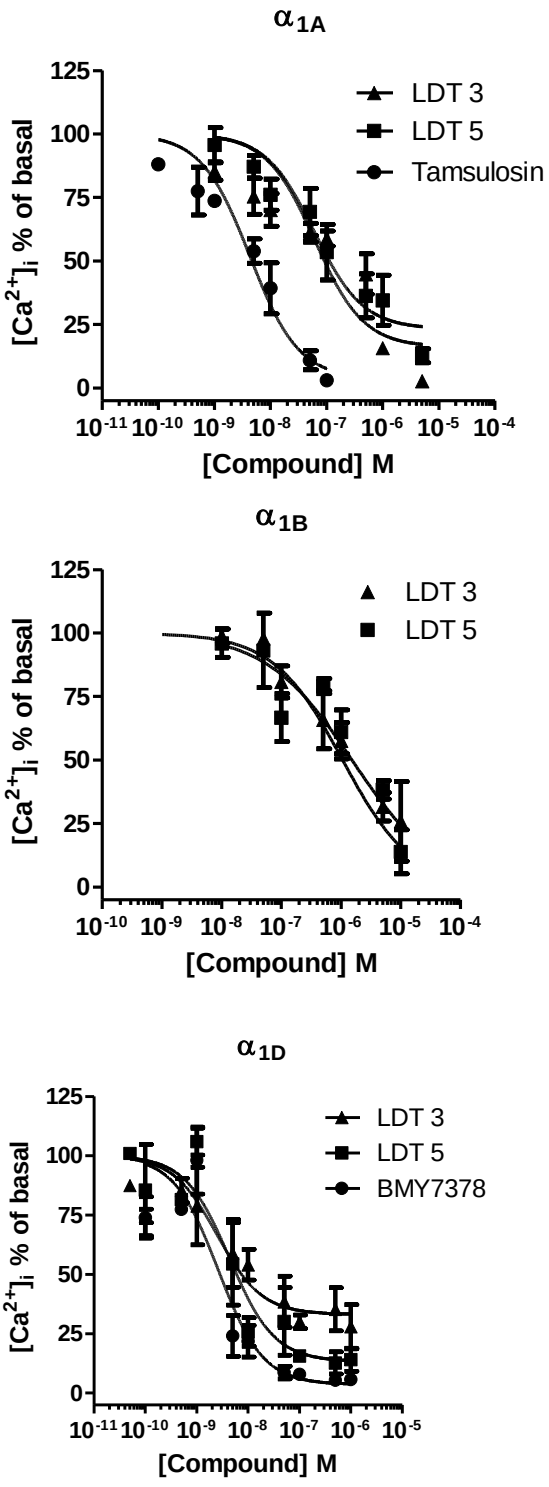
In these cells, proliferation (estimated by the MTT assay) induced by phenylephrine (A, PHE) or 5-HT (B) is mainly due to activation of α_{1D}-adrenoceptors and 5-HT_{1A} receptors, respectively. BMY 7378 and *p*-MPPF (α_{1D}-adrenoceptors and 5-HT_{1A} antagonists, respectively) were used as controls.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

$F_{7,32} = 7.558$, $P < 0.0001$ for α_{1D}-adrenoceptor. $F_{7,32} = 5.221$, $P = 0.0005$ for 5-HT_{1A} receptor. $^{***}P < 0.001$ vs. agonist alone. One-way analysis of variance (ANOVA) followed by the *post hoc* Dunnett's test.



Supplemental Figure 4. Inhibition of the growth of human DU-145 prostate cancer cells by LDT3, LDT5 and LDT8. Growth was estimated by MTT assay, and BMY 7378 (50 nM) and *p*-MPPF (50 nM) were used as selective antagonists of α_{1D}-adrenoceptors and 5-HT_{1A} receptors, respectively. In these cells, proliferation induced by phenylephrine (A, PHE) or 5-HT (B) is mainly due to activation of α_{1D}-adrenoceptors and 5-HT_{1A} receptors, respectively. Data are expressed as mean ± SD of 3-4 independent experiments performed in triplicates.

$F_{9,61} = 8.002$, $P < 0.0001$ for α_{1D}-adrenoceptor. $F_{9,61} = 5.394$, $P < 0.0001$ for 5-HT_{1A} receptor. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 compared to the agonists (one-way ANOVA followed by the *post hoc* Dunnett's test).



Supplemental Figure 5. LDT3 and LDT5 inhibit the increase of intracellular Ca^{2+} in rat-1 cells transfected with α_1-adrenocepotr subtypes. The increase of intracellular Ca^{2+} $[Ca^{2+}]i$ was induced by 100 μM phenylephrine. Antagonists were incubated for 100 sec before the addition of the agonist. Tamsulosin (n=3) and BMY7378 (n=3) were used as controls. LDT3 n=6 (α_{1D}), n=3 (α_{1A}, α_{1B}). LDT5 n=4 (α_{1D}), n=3 (α_{1A}, α_{1B}). Note that the error bars used here are the SEM (instead of SD) of the means.

References

Luthin GR, Wolfe BB (1984) [3H]Pirenzepine and [3H]quinuclidinyl benzilate binding to brain muscarinic cholinergic receptors. Differences in measured receptor density are not explained by differences in receptor isomerization. *Mol Pharmacol* **26**:164–169