European Journal of Medicinal Chemistry 46 (2011) 3000–3012

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Discovery of LASSBio-772, a 1,3-benzodioxole *N*-phenylpiperazine derivative with potent alpha 1A/D-Adrenergic receptor blocking properties

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ARTICLE INFO

Article history:
Received 27 November 2010
Received in revised form
4 April 2011
Accepted 12 April 2011
Available online 16 April 2011

Keywords:
N-Phenylpiperazine
Alpha-adrenoceptor antagonist
Benign prostatic hyperplasia
Safrole, 1,3-Benzodioxole

ABSTRACT

We described herein the discovery of 1-(2-(benzo[d] [1,3]dioxol-6-yl)ethyl)-4-(2-methoxyphenyl) piperazine (LASSBio-772), as a novel potent and selective alpha 1A/1D adrenoceptor (AR) antagonist selected after screening of functionalized *N*-phenylpiperazine derivatives in phenylephrine-induced vasoconstriction of rabbit aorta rings. The affinity of LASSBio-772 for alpha 1A and alpha 1B AR subtypes was determined through displacement of [^{3}H]prazosin binding. We obtained *Ki* values of 0.14 nM for the alpha 1A-AR, similar to that displayed by tamsulosin (K_i = 0.13 nM) and 5.55 nM for the alpha 1B-AR, representing a 40-fold higher affinity for alpha 1A-AR. LASSBio-772 also presented high affinity (K_B = 0.025 nM) for the alpha 1D-AR subtype in the functional rat aorta assay, showing to be equipotent to tamsulosin (K_B = 0.017 nM).

1. Introduction

The human adrenergic receptors are members of the G protein-coupled receptor superfamily that has been extensively exploited as targets for a great number of drugs useful in the treatment of many different diseases [1]. After the initial identification of the two main groups of adrenoceptors (AR), *i.e.* alpha-AR and beta-AR, their functional role has been progressively elucidated and their subclassification has been refined into alpha 1, alpha 2, beta 1, beta 2 and beta 3 [2,3].

Alpha 1-AR subtypes, *i.e.* alpha 1A, alpha 1B, and alpha 1D [4–6], have distinct pharmacology and tissue expression [7–9], a fact relevant for the treatment of several diseases, such as hypertension and the obstructive symptoms of the lower urinary tract [10], including the secondary urinary obstruction produced by benign prostatic hyperplasia (BPH) [8,11–13].

Alternatively to surgical procedures, alpha 1-AR antagonists are efficient to relief the obstructive symptoms of BPH by decreasing the prostatic muscular tonus, mainly through the blockage of alpha 1A-AR [14]. The drugs used for BPH treatment include the some quinazoline derivatives, initially with prazosin (**1**) and currently with terazosin (**2**), doxazosin (**3**) and alfuzosin (**4**) (Fig. 1), which are nonselective alpha 1-AR antagonists, so that hypotension triggered by alpha 1B-AR blockade is their main adverse effect [15,16].

On the other hand, the sulfonamide derivative tamsulosin (**5**) (Fig. 2), used clinically as a second generation therapeutic agent, has been reported to present a better uroselectivity profile due to its relatively higher affinity for alpha 1A and 1D subtypes [17]. However, its selectivity is modest and therefore is directly dependent on the therapeutic dosage (0.4 mg/day) ensuring the right balance between efficacy and safety [17–19].

In addition to the well characterized role of alpha AR in the obstructive symptoms of BPH, other studies demonstrated that both the alpha 1D and alpha 1A-AR subtypes are also expressed in

Abbreviations: AR, adrenoceptor; BPH, benign prostatic hyperplasia; EL, extracellular looping; HBA, hydrogen bond acceptor; MD, methylenedioxy; SL, spacer length; TM, transmembrane domain.

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doi:10.1016/j.ejmech.2011.04.032

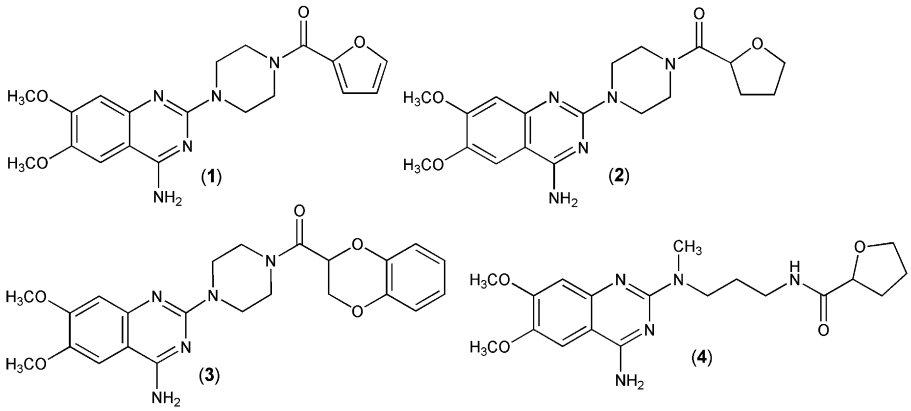


Fig. 1. Alpha-1 adrenoceptor antagonists clinically used for BPH treatment.

human detrusor muscle [20–22], and would be related to the irritating symptoms of BPH due to bladder flow obstruction. Taken together, these data suggest that the development of alpha 1A/alpha 1D-AR selective antagonists would indeed relief both obstructive and irritating symptoms of BPH, being the possible mechanism associated to tamsulosin (**5**) actions [8].

However, up to now, alpha-1-AR antagonists endowed with an ideal selectivity profile (*i.e.*, high affinity for alpha 1A/alpha 1D-AR but low affinity for alpha 1B-AR) are still in development [23–27], stimulating the search for new bioactive substances able to bypass the difficulties and disadvantages of the currently available pharmacological treatment of BPH.

In the scope of a research program aiming to design, synthesize and perform pharmacological evaluation of new selective alpha 1-AR antagonist lead-compound candidates for the treatment of secondary symptoms of BPH, we developed new functionalized 1,3-benzodioxolyl-*N*-phenylpiperazine derivatives **7a**–**k** (Fig. 2). These target compounds were designed by molecular hybridization of tamsulosin (**5**) and BMY7378 (**6**), selective alpha 1A and 1D antagonists, respectively, through the adequate combination of the pharmacophoric units Z, C, D and the nature and length of the spacer (SL) (Fig. 2), exploiting the Brazilian natural product safrole (**9**) as starting material (see structure in Scheme 1) [28]. In this case, based on the nature of the spacer we planned the isosteric oxa- and carbanalogue series, varying the number of methylene from 2 to 3 leading to a total spacer length (SL) of 3 (SL3) to 4 (SL4) by the inclusion of the oxygen atom in X for the first series, and from 1 (SL1) to 4 (SL4) for the second one. Additionally, the bis-oxygenated bridge attached to aryl subunit Z present in compounds of series **7** was introduced in order to mimic the hydrogen bond acceptor behavior of *ortho*-methoxysulfonamide subunit of **5** (a′ + a″, Fig. 2), fixing the orientation of the oxygen lone electron pairs. To evaluate the involvement of methylenedioxy bridge in the molecular recognition of **7** by alpha-1 AR we constructed the corresponding unsubstituted series **8**. Finally, in order to define structure–activity relationships based on stereoelectronic effects of substituents at *para*-position of the aryl subunit D we proposed the substitution of the hydrogen by fluorine, chlorine and methoxy groups (Fig. 2).

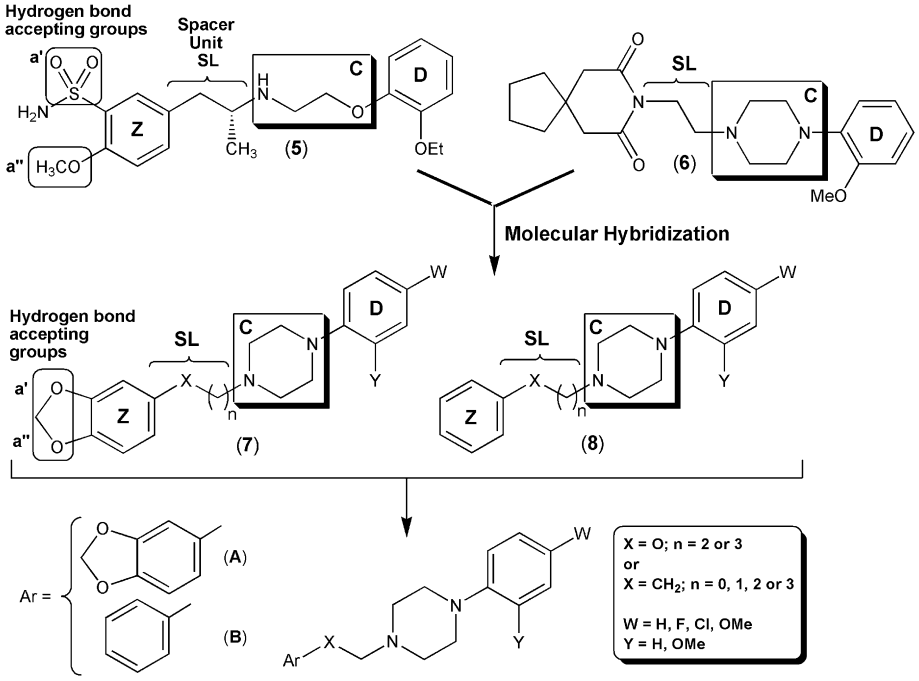
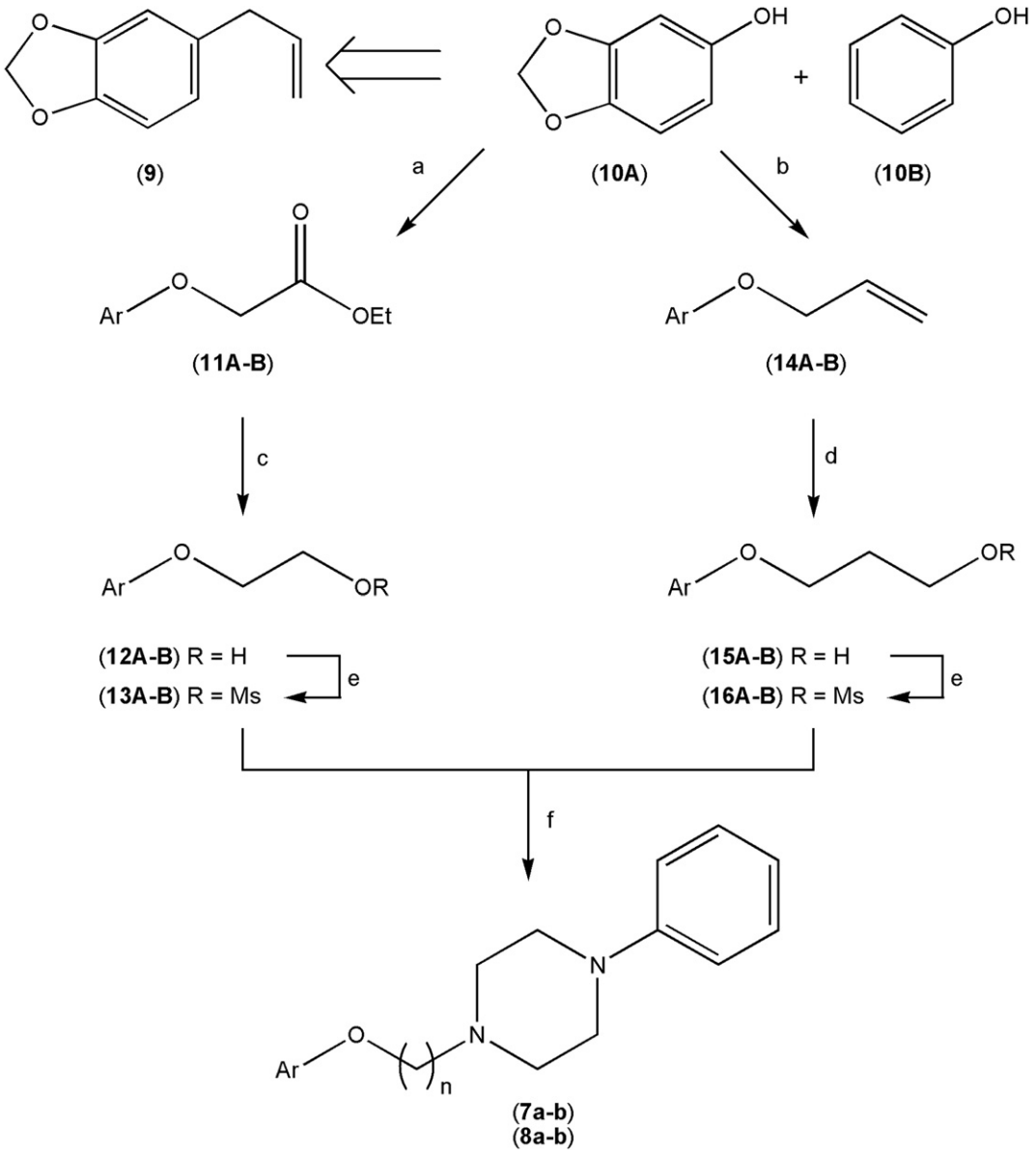


Fig. 2. Design concept of new functionalized 1,3-benzodioxolyl-*N*-phenylpiperazine derivatives (**7**) and unsubstituted phenyl analogues (**8**).



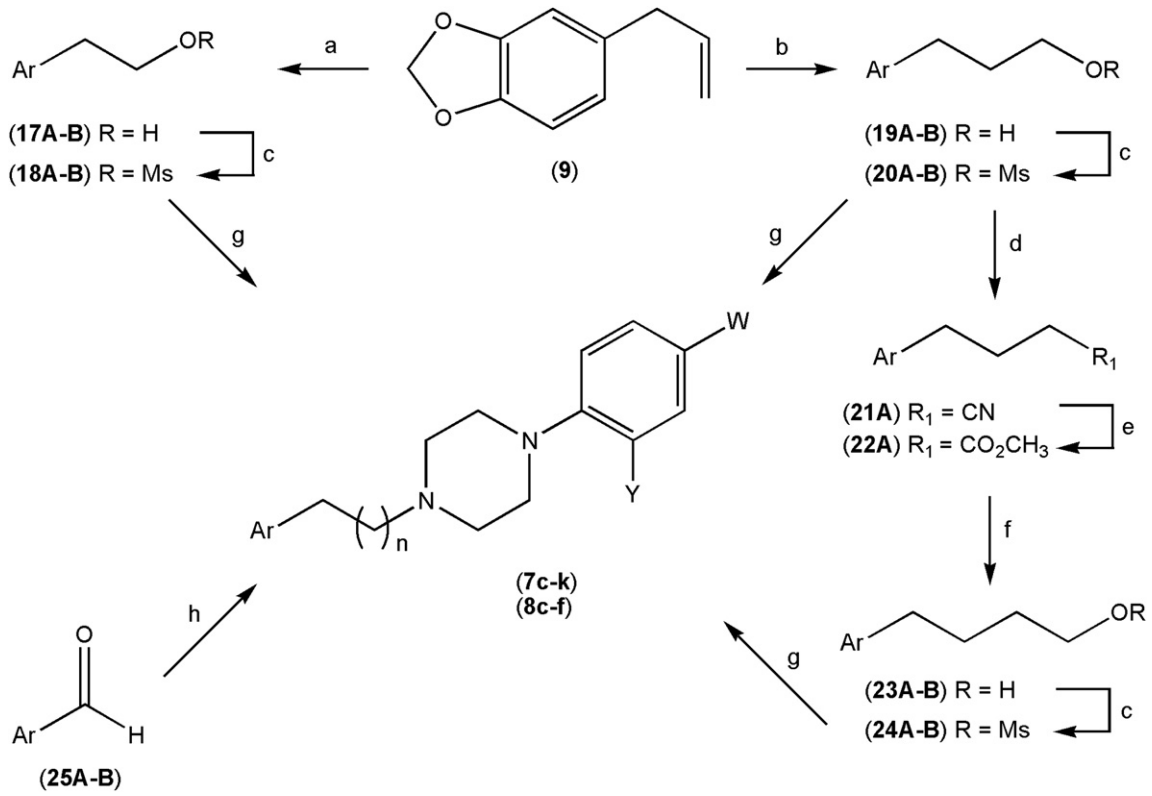
Scheme 1. Synthesis of aryl-*N*-phenylpiperazine oxanalogue derivatives (**7a**–**b**) and (**8a**–**b**). Ar(A) = 1,3-benzodioxolyl; Ar(B) = phenyl. Reagents and conditions: (a) K_2CO_3, acetone, $BrCH_2CO_2Et$, r.t., 3 h, 94% (**11A**) and 99% (**11B**); (b) K_2CO_3, acetone, $BrCH_2CH{=}CH_2$, r.t., 27 h, 98% (**14A**) and 83% (**14B**); (c) $LiAlH_4$, THF, r.t., 4 h, 96% (**12A**) and 88% (**12B**); (d) i: $BH_3.S(CH_3)_2$, CH_2Cl_2, r.t., 5 h, ii: 10% aq. NaOH, H_2O_2 30%, r.t., 20 h, 93% (**15A**) and 83% (**15B**); (e) MsCl, Et_3N, CH_2Cl_2, 0 °C → r.t., 3 h, 99% (**13A**), 90% (**13B**), 90% (**16A**) and 99% (**16B**); (f) *N*-phenylpiperazine, Li_2CO_3, CH_3CN, reflux, 24 h, 94% (**7a**), 92% (**7b**), 97% (**8a**) and 92% (**8b**).

2. Results and discussion

2.1. Chemistry

The novel series of 1,3-benzodioxole *N*-phenylpiperazine derivatives (**7**) were prepared by classical synthetic methodologies, exploiting sesamol (**10A**), piperonal (**25A**) and safrole (**9**)-derived compounds 2-(3,4-methylenedioxyphenyl)ethanol (**17A**) and 3-(3,4-methylenedioxyphenyl)propan-1-ol (**19A**) as starting material (Schemes 1 and 2). The corresponding unsubstituted analogs of series **8** were synthesized through equivalent methodologies, by using commercially available benzaldehyde (**25B**), 2-phenylethanol (**17B**), 3-phenylpropan-1-ol (**19B**) or 4-phenylbutan-1-ol (**23B**) as chemical precursors (Scheme 2). The initial step used for the construction of 1,3-benzodioxolyl-*N*-phenylpiperazine oxanalogue derivatives **7a** ($n = 2$) and **7b** ($n = 3$) consisted of the O-alkylation of **10A**–**B** with ethyl bromoacetate or allyl bromide as described previously [29,30], in order to introduce C-2 and C-3 in the spacer units leading to spacer length SL3 and SL4, respectively (Scheme 1). The reduction of acetate derivatives **11A**–**B** with lithium aluminum hydride in tetrahydrofuran furnished the C-2 alcohols **12A**–**B** in 96% and 88% yields, respectively. On the other hand, the *O*-allyl derivatives **14A**–**B** were then submitted to an oxidative hydroboration [31] to furnish the corresponding C-3 primary alcohols **15A**–**B** in respective 93% and 83% yields. Next, treatment of the alcohols **12A**–**B** and **15A**–**B** with mesyl chloride and triethylamine in dichloromethane furnished the corresponding mesylates **13A**–**B** and **16A**–**B**, which were converted in target derivatives **7a**–**b** and **8a**–**b** after nucleophilic displacement with *N*-phenylpiperazine in acetonitrile [32] (Scheme 1).

The 1,3-benzodioxolyl *N*-phenylpiperazine carbanalogue derivatives **7d** ($n = 1$) and **7e** ($n = 2$), presenting SL2 and SL3 respectively,



Scheme 2. Synthesis of aryl-*N*-phenylpiperazine carbanalogue derivatives (**7c**–**k**) and (**8c**–**f**). Ar(A) = 1,3-benzodioxolyl; Ar(B) = phenyl. Reagents and conditions: (a) i: O_3, -10 °C, CH_2Cl_2, 4 h, ii: $NaBH_4$, MeOH, 0 °C, 2 h; 72% (**17A**); (b) i: $BH_3.S(CH_3)_2$, CH_2Cl_2, r.t., 5 h, ii: 10% aq. NaOH, H_2O_2 30%, 60 °C, 14 h, 91% (**19A**); (c) MsCl, Et_3N, CH_2Cl_2, 0 °C → r.t., 3 h, 91% (**18A**), 99% (**18B**), 99% (**20A**) and 99% (**20B**) 98% (**24A**) and 99% (**24B**); (c) functionalized *N*-phenylpiperazine, Li_2CO_3, CH_3CN, reflux, 24 h (see experimental section); (d) NaCN, CH_3CN, reflux, 16 h, 99% (**21A**); (e) i: MeOH, HCl, r.t., 14 h, ii: H_2O, 0 °C, 20 min, 87%, (**22A**); (f) $LiAlH_4$, THF, r.t., 4 h, 89% (**23A**); (h) i: *N*-phenylpiperazine, K10, MW, 4 min, ii: $NaBH_4$, MeOH, r.t., 30 min, 90% (**7c**) and 98% (**8c**).

were prepared from 1,3-benzodioxolyl alcohols **17A** and **19A**, which were obtained from natural product **9** as previously reported [33–36].

On the other hand, the alcohol derivative **23A** was obtained in 76% yield (4 steps) from homologation of **19A** by applying the sequence of mesylation, nucleophilic displacement with cyanide anion [32], methanolysis in HCl [37,38], and reduction of the ester intermediate **22A** with lithium aluminum hydride in THF (Scheme 2).

The target derivatives **7d**–**f**, and **8d**–**f** were obtained in very good yields by nucleophilic substitution of the corresponding mesylates **18A**–**B**, **20A**–**B** and **24A**–**B** with *N*-phenylpiperazine in acetonitrile [32] (Scheme 2).

The shorter *N*-phenylpiperazine derivatives **7c** and **8c** were obtained, respectively, in 90% and 98% yields from reductive amination of the imine intermediate, generated by condensation of **25A**–**B** and *N*-phenylpiperazine under microwave irradiation [39], with sodium borohydride in methanol.

1,3-Benzodioxolyl derivatives **7g**–**k** functionalized at *ortho* or *para* positions of phenyl ring of *N*-phenylpiperazine moiety were prepared from mesylates **18A** and **20A**, as already described [32].

The structure of all seventeen *N*-phenylpiperazine derivatives of series **7** and **8** were in agreement with analytical and spectral data.

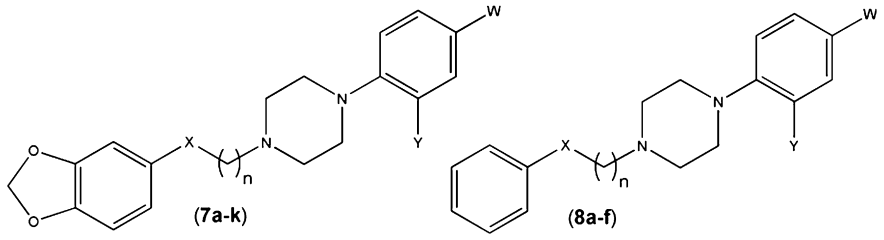
2.2. Pharmacology

The *N*-phenylpiperazine derivatives from both 1,3-benzodioxolyl **7** or phenyl **8** series were quantitatively transformed into water-soluble hydrochloride salts and initially evaluated as inhibitors of the phenylephrine-induced constriction of rabbit aorta rings [40]. As reported in Table 1, most of the compounds displaced the phenylephrine concentration–response curve to the right, as the (log) EC_{50} values increased in their presence, which is also suggestive of a competitive antagonism. Note that we used a single concentration for all antagonists and not a wide range of concentrations which is necessary for Schild regression analysis. However such protocol enabled to estimate the dissociation constant of the antagonists (K_B) through the Schild equation [41] (see experimental section). With the exception of LASSBio-772 (**7g**: K_B = 18 nM) all derivatives have a low affinity (μM range) for the alpha AR expressed in rabbit aorta (mainly alpha 1A) [42] (Table 1).

Concerning the structural differences among the derivatives we can draw some conclusions about three aspects of the structure–activity relationship: i. the influence of the spacer atom and length of the alkyl chain between the aromatic units Z and D; ii. the importance of electronic and hydrophobic interactions with the aromatic unit Z; and iii. the influence of stereoelectronic factors of substituents at *ortho* and *para* positions of the phenylpiperazine moiety.

With respect to the influence of the spacer unit between the phenylpiperazine moiety and the aromatic unit Z, the bioisosteric replacement of the oxygen atom in the alkyl chain (LASSBio-629, **7a** and LASSBio-670, **7b**) by methylene group in LASSBio-632 (**7e**) and LASSBio-675 (**7f**) has a positive influence on the affinity for the alpha 1-AR, as indicated by an increase in the potency of the corresponding carbanalogues **7e** and **7f** (Table 1). The distinct biological profile of LASSBio-629 (**7a**) and LASSBio-632 (**7e**) may be

Table 1
Structures and affinities for rabbit aorta alpha 1-AR of new 1,3-benzodioxolyl *N*-phenylpiperazine derivatives (**7a**–**k**) and their unsubstituted analogues (**8a**–**f**).



Compound	X	n	SL	Y	W	Phenylephrine log EC_{50} (M)[a]	K_B (μM)[b]
Control	–	–	–	–	–	−6.189 ± 0.101	–
BMY7378 (**6**)	–	–	–	–	–	−4.851 ± 0.087	1.45
LASSBio-629 (**7a**)	O	2	3	H	H	−5.592 ± 0.087	10.3
LASSBio-670 (**7b**)	O	3	4	H	H	−5.870 ± 0.088	27.5
LASSBio-719A (**7c**)	CH_2	0	1	H	H	−5.850 ± 0.178	25
LASSBio-680 (**7d**)	CH_2	1	2	H	H	−4.145 ± 0.022	0.27
LASSBio-632 (**7e**)	CH_2	2	3	H	H	−5.240 ± 0.032	3.8
LASSBio-675 (**7f**)	CH_2	3	4	H	H	−5.560 ± 0.135	9.38
LASSBio-772 (**7g**)	CH_2	1	2	OCH_3	H	−2.967 ± 0.032	0.018
LASSBio-773 (**7h**)	CH_2	2	3	OCH_3	H	−3.821 ± 0.025	0.13
LASSBio-681 (**7i**)	CH_2	1	2	H	F	−4.315 ± 0.019	0.41
LASSBio-682 (**7j**)	CH_2	1	2	H	Cl	−4.787 ± 0.026	1.24
LASSBio-683 (**7k**)	CH_2	1	2	H	OCH_3	−4.638 ± 0.027	0.87
LASSBio-728 (**8a**)	O	2	3	H	H	−5.479 ± 0.038	7.32
LASSBio-729 (**8b**)	O	3	4	H	H	−6.228 ± 0.041	ND
LASSBio-719B (**8c**)	CH_2	0	1	H	H	−5.847 ± 0.167	25
LASSBio-771 (**8d**)	CH_2	1	2	H	H	−4.852 ± 0.072	1.46
LASSBio-724 (**8e**)	CH_2	2	3	H	H	−5.566 ± 0.055	9.38
LASSBio-730 (**8f**)	CH_2	3	4	H	H	−4.842 ± 0.015	1.42

SL: total spacer length.
[a] Values are mean ± S.E.M of 4–6 experiments.
[b] K_B values were calculated by applying the Schild equation [41] (see experimental section).

related to a better hydrophobic recognition of the spacer unit or associated to the different electronic behavior introduced in the ring Z by the insertion of a heteroatom in the spacer (Fig. 2), influencing the recognition of 1,3-benzodioxole ring by complementary amino acids residues at the target alpha 1-AR, *i.e.* Trp-165 and Tyr-184 (TM-4 and TM-5, respectively (alpha 1A)), Trp-184 and Tyr-203 (EL and TM-5, respectively (alpha 1B)), Trp-235 and Tyr-254 (TM-4 and TM-5, respectively (alpha 1D)), as previously suggested by Bautista and colleagues [43].

Since carbanalogues in general showed higher affinity than their corresponding oxanalogues for alpha 1-AR in rabbit aorta (mainly alpha 1A-AR), we decided to evaluate the influence of the hydrophobic spacer length in this series, which varied from zero to three methylene units ($n = 0$ to 3) corresponding to SL1 to SL4 (Table 1). On the other hand, taking into account that the spacer is responsible for the distance between the pharmacophoric phenylpiperazine (C + D) and the auxophoric aromatic Z (Fig. 2) moieties, we consider that the modulation of the antagonist activity could be related to recognition of the 1,3-benzodioxole moiety, especially to the methylenedioxy (MD) subunit as hydrogen bond acceptor group (HBA). In this case, when the MD subunit is shifted away it may interact with the adequate amino acids residues between transmembrane domains (TM) of target alpha 1-AR at the antagonist binding pocket closer to the extracellular surface, above the plane of the binding site for agonists [44,45].

The influence of electronic interactions with the aromatic subunit Z was investigated through the comparison of 1,3-benzodioxolyl (**7**) and phenyl (**8**) series, which helped us to identify the relevance of the presence of an HBA group represented by the MD subunit for the molecular recognition by target alpha 1-AR. The results displayed in Table 1 revealed that compounds (**7c**) and (**8c**) with a methylene group in the spacer unit ($n = 0$, SL1) had the lowest affinity for alpha 1-AR (K_B value ~ 25 μM). Our findings are in agreement with those described by López-Rodríguez and collaborators [46,47] in which the influence of the alkyl chain size in the modulation of the alpha 1-AR affinity seems to vary from 2 to 4 methylenes in the spacer. Regarding the carbanalogues of the 1,3-benzodioxololyl series (**7**), we found that the affinity (**7d** > **7e** > **7f**) decreases when increasing the length of the spacer from 2 to 4 methylene units (Table 1). On the other hand, in the series of the *N*-phenylpiperazine derivatives (**8**), which have essentially hydrophobic/aromatic features, the influence of the spacer length ($n = 1$–3, SL2-4) is not so straightforward, with a higher affinity when SL2 or SL4: (**8d**) (K_B 1.46 μM) ≅ (**8f**) (K_B 1.42 μM) > (**8e**) (K_B 9.38 μM) (Table 1). As a general trend, *N*-phenylpiperazine derivatives of the series (**7**), presenting the MD subunit, displayed a better antagonist activity than those of the series (**8**), except for the compounds **8f** with SL4.

In order to analyze the influence of stereoelectronic factors of the substituents in the phenylpiperazine moiety, we decided to synthesize new compounds with a methoxy group at the *ortho* position since the alkoxy fragments seem to play an important role on the structural feature of some alpha 1-AR antagonists, *e.g.* tamsulosin (**5**) and BMY7378 (**6**), through particular interactions with aromatic amino acids located at TM-3, TM-6 and TM-7 of these receptors [43,48]. So, the new compounds (**7g**) and (**7h**) were synthesized and used as hydrochloride salts in the same functional assay (rabbit aorta). This structural change increased the affinity of the molecule with spacer SL2 (**7g**, K_B 0.018 μM vs. **7d**, K_B 0.27 μM) and SL3 (**7h**, K_B 0.13 μM vs. **7e**, K_B 3.8 μM) (Table 1). The comparison

between derivatives (**7g**) and (**7h**) further reinforce the influence of the adequate spacer length in the modulation of the alpha 1-AR antagonist affinity, with the alkyl chain composed by two methylene units (SL2, Fig. 1) allowing a better recognition by the target alpha 1-AR. Amongst the derivatives studied, LASSBio-772 (**7g**) and LASSBio-773 (**7h**) have the highest affinities, the first being *ca.* 80 times more potent than BMY7378 (**6**) used as standard drug (Table 1).

Therefore, due to the significant blockage of phenylephrine-induced contraction of *N*-(2-methoxyphenyl)piperazine derivative (**7g**), we decided to investigate the influence of fluoro, chloro and methoxy groups at the *para*-position of the *N*-phenylpiperazine moiety for the 1,3-benzodioxolyl carbanalogue derivatives with spacer length 2 (SL2). All the *para*-substituted compounds (**7i**–**k**) exhibited, comparatively to unsubstituted *N*-phenylpiperazine derivative LASSBio-680 (**7d**), reduction of the affinity (Table 1). However, the derivative **7i** (W = F) has a relatively low K_B (0.41 μM) comparing with the ones of (**7j**) (R = Cl, K_B 1.24 μM) and (**7k**) (R = OMe, K_B 0.87 μM). In this case, derivatives presenting *para*-substituents such as chloro and methoxy group, independent of the influence on the electronic or hydrophobic interactions for alpha 1-AR, showed a decrease in activity, which may be due to an unfavorable steric interaction. These findings are in agreement with the hypothesis of a steric restriction in the alpha 1-AR cavity delimiting the volume accessible to the ligands presenting a *para*-substituent in the phenylpiperazine subunit so that the bioprofile of these derivatives is modulated mainly by steric factors [47]. The decrease in activity of the *para*-fluoro derivative (**7i**), may be related to the inductive and mesomeric effects of the group, changing the electronic density distribution on the aromatic ring, which interacts with complementary aminoacid residues of the recognition site at the target alpha 1-AR.

Due to its relevant blockage of phenylephrine-induced contraction of rabbit aorta, LASSBio-772 (**7g**) was selected to further evaluate its affinity for different subtypes of alpha 1-AR, through displacement of [^{3}H]prazosin specific binding in rat salivary gland (alpha 1A) and liver (alpha 1B). The binding data showed that LASSBio-772 (**7g**) has a *Ki* value of 0.14 nM (Table 2) for the alpha 1A and 5.55 nM for the alpha 1B-AR subtypes (Fig. 3, Table 2), therefore presenting a high affinity for the alpha 1A-AR, which is similar to the affinity of tamsulosin (*Ki* = 0.13 nM) [49], the first antagonist approved in the United States for the treatment of BPH. Similar results were obtained in native alpha 1A-AR expressed in rabbit liver (*data not shown*). With regard to the K_B value (18 nM) observed in functional assays with rabbit aorta some points must be considered. First, in thoracic rabbit aorta although alpha 1A-AR subtype is the predominant one, for both extension of expression and functional relevance, alpha 1B and 1D subtypes are also expressed [50–52] so that we cannot rule out that this estimation of K_B value of LASSBio-772 reflects the blockage of a mixture of AR subtypes rather than only alpha 1A-AR. Second, all subtypes of alpha 1-AR induce vascular contraction, but the alpha 1A-AR is related to a more efficient increase in the intracellular calcium and sensitization of contractile machinery [52], which may also explain the smaller potency of the antagonist observed in the functional assay, where the activated receptor is linked to the signaling pathway, as compared to the binding assay, where we only determine the affinity of the ligand for the receptor.

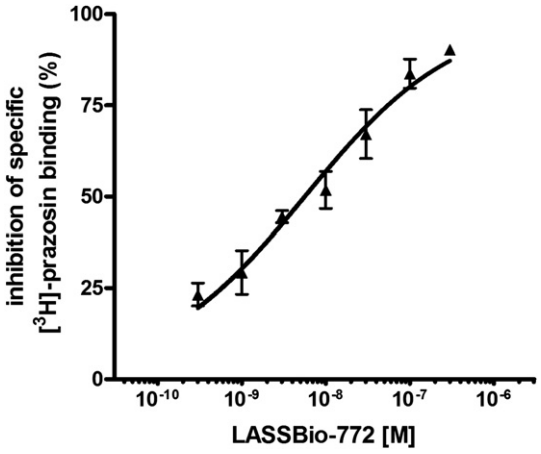


Fig. 3. Competition curve of LASSBio-772 in preparation enriched in native α_{1B}-adrenoceptors (rat liver membranes). Data are expressed as mean ± SEM of individual experiments ($n = 3$).

On the other hand, it should be highlighted that LASSBio-772 (**7g**) has a 40-fold higher affinity for alpha 1A over alpha 1B-AR, whereas this affinity ratio was only 14.8-fold for tamsulosin (**5**) [49], indicating that this new 1,3-benzodioxolyl *N*-phenylpiperazine derivative exhibits a slightly higher selectivity for alpha 1A versus alpha 1B-AR than tamsulosin.

In order to evaluate the antagonist activity of LASSBio-772 (**7g**) for the alpha 1D subtype, we performed functional assays in rat thoracic aorta. In this tissue alpha 1D-AR mRNA comprises over 85% of the total alpha 1-AR mRNA, and therefore it is considered as a tissue enriched in this subtype of alpha 1-AR [53]. In this model, prazosin (**1**) had a mean K_B value of 0.17 nM (Table 2), which is compatible with the literature [54]. Additionally, the selective alpha 1D-AR antagonist BMY7378 (**6**), also used as positive control, had a mean K_B value of 3 nM (Table 2) similar to the one previously described ($K_B = 6$ nM) in

Table 2
Pharmacological parameters of LASSBio-772 (**7g**), prazosin and BMY7378 in native rat alpha 1-AR.

Binding studies	−log IC_{50}[a]	*Ki* (nM)
LASSBio-772; alpha 1B-AR (rat liver)	8.22 ± 0.14 ($n = 3$)	5.55
Prazosin; alpha 1B-AR (rat liver)	9.07 ± 0.19 ($n = 3$)	0.55
LASSBio-772; alpha 1A-AR (rat salivary gland)	9.57 ± 0.05*	0.14
Functional studies	pA_2	K_B (nM)[b]
LASSBio-772; alpha 1D-AR (rat aorta)	10.6 ± 0.2 ($n = 8$)	0.025
Prazosin; alpha 1D-AR (rat aorta)	9.77 ± 0.06 ($n = 3$)	0.17
BMY7378; alpha 1D-AR (rat aorta)	8.52 ± 0.06 ($n = 5$)	3.02

*Data from one experiment performed in triplicate at CEREP facilities (France). In this case, the standard error assesses the precision of the best fit value for the experiment.

[a] Parameters obtained by non-linear regression (sigmoidal dose-response equation) are expressed as mean and S.E.M. of n individual experiments. K_i values were calculated using the Cheng and Prusoff equation. For prazosin binding at the α_{1B}–adrenoceptor, the K_d value was calculated from saturation experiments.

[b] K_B was determined through Schild regression (see methods; regression analysis: $R^2 = 0.97$).

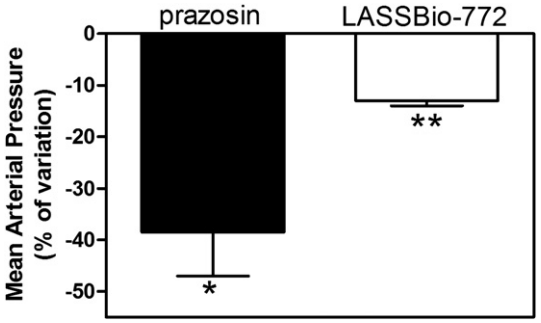


Fig. 4. Rabbit mean arterial pressure variation induced by prazosin or LASSBio-772 (100 μg/kg, i.v.). *P and **$P < 0.05$ vs. control (0%) and prazosin, respectively, $n = 5$, Student's t test. The absolute control value was 90 ± 4 mmHg ($n = 5$).

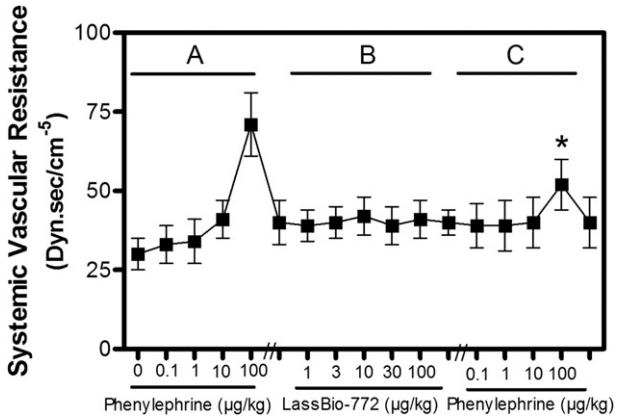


Fig. 5. Phenylephrine-induced rabbit systemic vascular resistance increase before (A) and after LASSBio-772 treatment (100 µg/kg, in bolus) (C). B. Infusion of cumulative doses of LASSBio-772 (1–100 µg/kg). *$P < 0.05$ vs. phenylephrine 100 µg/kg before LASSBio-772 treatment (100 µg/kg), $n = 5$, paired Student's t test. A, B and C represent different experimental conditions separated by//.

the same model [55]. LASSBio-772 (**7g**) induced a parallel displacement of the agonist curve to the right, in a concentration-dependent manner, and also exhibited a high potency to inhibit rat aorta contraction with an affinity in the subnanomolar range ($K_B = 0.025$ nM). A similar value of K_B (0.054 nM) was observed in the absence of functional endothelium ($pK_B = 10.27 \pm 0.29$, $n = 6$) discarding any interference of endothelium-derived mediators such as the vasodilator nitric oxide. Therefore LASSBio-772 is equipotent to tamsulosin ($K_B = 0.017$ nM) [56]. When compared to **6**, LASSBio-772 (**7g**) was about 120-fold more potent, confirming its excellent alpha 1D-AR antagonist activity.

Considering that LASSBio-772 (**7g**) showed an alpha 1-AR antagonistic action *in vitro*, we decided to evaluate its effect *in vivo*. As shown in Fig. 4, when analyzing the impact of LASSBio-772 (**7g**) upon arterial blood pressure as compared to a reference nonselective alpha 1-AR antagonist (prazosin), both at the same dose (100 µg/kg), we observed that the hypotensive effect of LASSBio-772 (**7g**) was significantly smaller than the one induced by prazosin. In addition, the full range of doses of LASSBio-772 (**7g**) did not alter the systemic vascular resistance (Fig. 5), reinforcing its small impact in vascular physiology probably due to the relatively smaller affinity for alpha 1B-AR, normally involved in blood pressure control [57]. However, the highest dose (100 µg/kg) of LASSBio-772 (**7g**) blunted the vasoconstriction action of the exogenous alpha 1-AR agonist phenylephrine (0.1–100 µg/kg) confirming its antagonism on alpha 1-AR *in vivo* (Fig. 5).

3. Conclusion

As concluding remarks, we described herein the discovery of 1-(2-(benzo[d] [1,3]dioxol-6-yl)ethyl)-4-(2-methoxyphenyl)piperazine (LASSBio-772, **7g**) as a novel alpha 1 adrenoceptor antagonist. This compound presents pharmacological features higher affinity for the alpha 1A/1D than alpha 1B-AR, being therefore putatively useful for the treatment of the lower urinary tract symptoms, including the benign prostatic hyperplasia in mammals [58].

4. Experimental protocols

4.1. Instrumentation, chemicals and solvents

Melting points were determined on a Quimis 340/23 apparatus and are uncorrected. Infrared (IR) spectra were obtained on a Nicolet-Magna 760 infrared spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 200 instrument. Chemical shifts (δ) are expressed in parts per million relative to internal tetramethylsilane; coupling constants (*J*) are in hertz. Microanalyses were obtained with Thermofinnigan EA1112 analyzer, using a Metler MX5 electronic balance. Reactions were routinely monitored by thin-layer chromatography (TLC) in silica gel (F254 Merck plates) and the products visualized with iodine or ultraviolet lamp (254 and 365 nm). For normal pressure and flash column chromatography purifications, Merck silica gel type 60 (size 70–230 and 230–400 mesh, respectively) was used. Unless stated otherwise, starting materials used were high-grade commercial products.

4.1.1. General procedure for preparation of alcohols (*12A–B*)

To a suspension of lithium aluminum hydride (0.249 g, 6.55 mmol) in anhydrous THF (40 mL) was added during 15 min a solution of the corresponding β-oxoester derivative (**11A**) or (**11B**) (3.12 mmol) in anhydrous THF (20 mL). After 4 h, the excess of reductive agent was quenched with methanol (1 mL) and 10% aqueous NaOH solution (1 mL) until formation of aluminum hydroxide, which was neutralized with 10% aqueous hydrochloric acid solution (*ca.* 2 mL). The obtained mixture was extracted with ethyl acetate (3 × 25 mL) and the combined organic extracts were washed with brine and concentrated at reduced pressure after drying with anhydrous sodium sulfate. The residue was separated by chromatography on silica gel eluted with chloroform to give the corresponding primary alcohols (**12A**) and (**12B**), as described in Supplementary Material.

4.1.2. General procedure for preparation of alcohols (*15A–B*)

To a solution of the corresponding allyl ethers (**14A**) or (**14B**) (3.92 mmol) in dichloromethane (35 mL) was added a 1M borane–dimethylsulfide solution in dichloromethane (5.5 mL, 5.50 mmol). The mixture was vigorously stirred at room temperature, under nitrogen atmosphere and protected from light. After 5 h, the excess of reductive agent was quenched with methanol (1 mL) and 10% NaOH solution (3 mL) and 30% aqueous H_2O_2 (3 mL). The suspension was stirred at room temperature for 20 h. The mixture was extracted with dichloromethane (3 × 25 mL) and the combined organic extracts were washed with 10% aqueous hydrochloric acid solution, and brine. After drying under sodium sulfate and evaporation of the solvent under reduced pressure, the residue was purified by silica gel column chromatography after elution with hexane–chloroform 1:1 to give the corresponding primary alcohols (**14A**) and (**14B**), as described in Supplementary Material.

4.1.3. Preparation of 4-(benzo[d] [1,3]dioxol-5-yl)butanenitrile (*21A*)

A solution of the mesylate (**20A**) (0.130 g, 0.50 mmol) and NaCN (0.074 g, 1.50 mmol) in acetonitrile (4 mL) was stirred at reflux for 16 h under nitrogen atmosphere. At the end of this time the solution was cooled to room temperature, poured into ice-water mixture and extracted with dichloromethane (3 × 15 mL). The combined organic extracts were successively washed with saturated aqueous Na_2CO_3 solution (2 × 5 mL), 10% aqueous hydrochloride solution (2 × 15 mL) and brine (10 mL). After drying over anhydrous sodium sulfate and evaporation of the solvent under reduced pressure, the residue was chromatographed on silica gel eluted with chloroform to give 0.072 g (99%) of the corresponding nitrile (**21A**), as described in Supplementary Material.

4.1.4. Preparation of methyl 4-(benzo[d] [1,3]dioxol-5-yl)butanoate (*22A*)

To a solution of nitrile derivative (**21A**) (0.167 g, 0.88 mmol) in methanol (25 mL) was slowly bubbled with hydrogen chloride until

saturation. The mixture was stirred at room temperature until the reaction shown to be complete after 14 h. At the end of this time cold water (20 mL) was added. The solvent was concentrated until half of the initial volume and the mixture was extracted with ethyl acetate (3 × 30 mL). The combined organic extracts were washed with brine (20 mL). After drying over anhydrous sodium sulfate and evaporation of the solvent under reduced pressure, the residue was separated by chromatography on silica gel eluted with chloroform to give 0.171 g (87%) of the corresponding methyl ester (**22A**), as described in Supplementary Material.

*4.1.5. Preparation of 4-(benzo[d] [1,3]dioxol-5-yl)butan-1-ol (**23A**)*

A solution of methyl ester (**22A**) (0.250 g, 2.25 mmol) in anhydrous THF (40 mL) was added during 30 min to a suspension of lithium aluminum hydride (6.58 mmol) in anhydrous THF (40 mL). After 4 h, the excess of reductive agent was quenched by addition of methanol (1 mL) and 10% aqueous NaOH solution (6 mL) until formation of aluminum hydroxide, which was neutralized with 10% aqueous hydrochloric acid solution (8 mL). The mixture was extracted with ethyl acetate (3 × 25 mL) and the combined extracts were washed with brine. After drying over anhydrous sodium sulfate and evaporation of the solvent under reduced pressure, the obtained residue was separated by chromatography on silica gel eluted with chloroform to give 0.390 g (89%) of the alcohol (**23A**), as described in Supplementary Material.

*4.1.6. General procedure for the preparation of mesylates (**13A**–**B**), (**16A**–**B**), (**18A**–**B**), (**20A**–**B**), (**24A**–**B**)*

To a solution of corresponding alcohol (**12A**–**B**) or (**15A**–**B**) or (**17A**–**B**) or (**19A**–**B**) or (**23A**–**B**) (2.14 mmol) and triethylamine (0.36 mL, 2.59 mmol) in anhydrous dichloromethane (6 mL), kept to 0 °C, was added mesyl chloride (0.18 mL, 2.31 mmol). The reaction was maintained at 0 °C during the first hour, followed by warming to room temperature, under vigorous stirring and nitrogen atmosphere for 3 h. At the end of this time the solution was extracted with dichloromethane (3 × 30 mL) and the combined organic extracts were washed with a 10% aqueous hydrochloric acid solution, brine, dried over sodium sulfate and evaporated. The obtained residue was separated by chromatography on silica gel eluted with dichloromethane, followed by chloroform to give the desired O-mesylated derivatives, as described in Supplementary Material.

*4.1.7. General procedure for preparation of functionalized N-phenylpiperazine derivatives (**7a**–**b**), (**7d**–**k**), (**8a**–**b**) and (**8d**–**f**)*

To a suspension of corresponding mesylate derivative (**13A**–**B**), (**16A**–**B**), (**18A**–**B**), (**20A**–**B**), (**24A**–**B**) (0.50 mmol) and lithium carbonate (0.07 g, 1.00 mmol) in acetonitrile (6 mL), was added the adequate *N*-phenylpiperazine derivative (2.00 mmol). The mixture was kept at reflux for 24 h under vigorous agitation and nitrogen atmosphere. At the end of this time the solution was concentrated in a rotatory evaporator, solubilized in dichloromethane and mixed with silica gel. The material was separated by chromatography on silica gel eluted with dichloromethane, followed by chloroform to give the desired *N*-phenylpiperazine derivatives of 1,3-benzodioxolyl (**7**) and phenyl (**8**) series, as described next.

*4.1.7.1. 1-(2-(Benzo[d] [1,3]dioxol-5-yloxy)ethyl)-4-phenylpiperazine (LASSBio-629, **7a**).* Prepared from nucleophilic substitution of mesylate (**13A**) with *N*-phenylpiperazine, as a white solid; 0.154 g (94%); Rf = 0.58 ($CHCl_3$:EtOH 5%); mp 95–96 °C; IR (KBr, cm^{-1}) 3035, 2921, 2843, 1632, 1598, 1503, 1479, 1311, 1190, 1031; ^1H NMR ($CDCl_3$): δ 2.72 (m, 4H, $ArNCH_2CH_2N$), 2.82 (t, *J* = 5.59 Hz, 2H, OCH_2CH_2N), 3.21(m, 4H, $ArNCH_2CH_2N$), 4.05 (t, *J* = 5.59 Hz, 2H, OCH_2CH_2N), 5.89 (s, 2H, OCH_2O), 6.33 (dd, J^2 = 8.61 Hz, J^3 = 1.93, 1H, Ar–H-6′), 6.51 (d, J^3 = 1.93 Hz 1H, Ar–H-2′), 6.69 (d, J^2 = 8.61 Hz, 1H, Ar–H-5′), 6.82 (m, N–Ar–H-4″), 6.92 (m, N–Ar–H-2″), 7.25 (m, N–Ar–H-3″); ^{13}C NMR ($CDCl_3$): δ 48.9 ($ArNCH_2CH_2N$), 53.4 (NCH_2CH_2O), 57.0 ($ArNCH_2CH_2N$), 66.7 (NCH_2CH_2O), 98.0 (Ar-2′-CH), 100.9 (OCH_2O), 105.6 (Ar-5′-CH), 107.7 (Ar-6′-CH), 115.8 (Ar-2″ e 6″- 2CH), 119.5 (Ar-4″-CH), 128.9 (Ar-3″ e 5″-2CH), 141.5 (Ar-4′-C), 148.0 (Ar-3′-C), 151.0 (Ar-1″-C–N), 154.0 (Ar-1′-C–O). Anal. Calcd. for $C_{19}H_{22}N_2O_3$: C, 69.92; H, 6.79; N, 8.58. Found: C, 70.11; H, 6.77; N. 8.55.

*4.1.7.2. 1-(3-(Benzo[d] [1,3]dioxol-5-yloxy)propyl)-4-phenylpiperazine (LASSBio-670, **7b**).* Prepared from nucleophilic substitution of mesylate (**16A**) with *N*-phenylpiperazine, as a light-beige solid; 0.123 g (92%); Rf = 0.45 ($CHCl_3$:EtOH 5%); mp 80–82 °C; IR (KBr, cm^{-1}) 3035, 2950, 2819, 1633, 1600, 1501, 1447, 1244 1181, 1036; ^1H NMR ($CDCl_3$): δ 1.92–2.03 (m, 2H, $OCH_2CH_2CH_2N$), 2.51–2.63 (m, 6H, $OCH_2CH_2CH_2N$ (2H) e $ArNCH_2CH_2N$ (4H)), 3.17–3.22 (m, 4H, $ArNCH_2CH_2N$), 3.95 (t, *J* = 6.1 Hz, 2H, $OCH_2CH_2CH_2N$), 5.88 (s, 2H, OCH_2O), 6.32 (dd, J^3 = 8.4 Hz, J^4 = 2.0 Hz, 1H, Ar– H-6′), 6.50 (d, J^4 = 2.0 Hz, 1H, Ar–H-2′), 6.69 (d, J^3 = 8.4 Hz, 1H, Ar–H-5′), 6.80–6.94 (m, 3H, N–Ar–H-2″, 4″ e 6″), 7.21–7.29 (m, 2H, N–Ar–H-3″ e 5″); ^{13}C NMR ($CDCl_3$): δ 26.6 ($OCH_2CH_2CH_2N$) 48.9 (NCH_2CH_2NAr), 53.1 (NCH_2CH_2NAr), 55.0 ($OCH_2CH_2CH_2N$), 67.0 ($OCH_2CH_2CH_2N$), 97.9 (Ar-2′-CH), 100.9 (OCH_2O), 105.5 (Ar-5′-CH), 107.7 (Ar-6′-CH), 115.8 (Ar-2″ e 6″- 2CH), 119.5 (Ar-4″-CH), 128.9 (Ar-3″ e 5″-2CH), 141.3 (Ar-4′-C), 148.0 (Ar-3′-C), 151.1 (Ar-1″-C–N), 154.0 (Ar-1′-C–O). Anal. Calcd. for $C_{20}H_{24}N_2O_3$: C, 70.56; H, 7.11; N, 8.23. Found: C, 70.68; H, 7.10; N. 8.19.

*4.1.7.3. 1-(2-(Benzo[d] [1,3]dioxol-5-yl)ethyl)-4-phenylpiperazine (LASSBio-680, **7d**).* Prepared from nucleophilic substitution of mesylate (**18A**) with *N*-phenylpiperazine, as a white solid; 0.113 g (86%); Rf = 0.57 ($CHCl_3$:EtOH 5%); mp 85–86 °C; IR (KBr, cm^{-1}) 3019, 2943, 2822, 1598, 1489, 1441, 1337, 1237, 1193, 1152, 1041; ^1H NMR ($CDCl_3$): δ 2.54–2.80 (m, 8H, $ArCH_2CH_2N$ (4H) e NCH_2CH_2NAr (4H)) 3.18–3.22 (m, 4H, NCH_2CH_2NAr), 5.88 (s, 2H, OCH_2O), 6.65 (dd, J^3 = 7.8 Hz, J^4 = 1.6 Hz, 1H, Ar– H-6′), 6.71 (s, 1H, Ar–H-2′), 6.73 (d, J^3 = 7.8 Hz, 1H, Ar–H-5′), 6.80–6.94 (m, 3H, N–Ar–H-2″, 4″ e 6″), 7.20–7.30 (m, 2H, N–Ar–H-3″ e 5″); ^{13}C NMR ($CDCl_3$): δ 33.1 ($ArCH_2CH_2N$), 48.9 (NCH_2CH_2NAr), 53.0 (NCH_2CH_2NAr), 60.4 ($ArCH_2CH_2N$), 100.5 (OCH_2O), 107.9 (Ar-2′-CH), 108.9 (Ar-5′-CH), 115.8 (Ar-2″ e 6″- 2CH), 119.4 (Ar-6′-CH), 121.2 (Ar-4″-CH), 128.8 (Ar-3″ e 5″-2CH), 133.8 (Ar-1′-C), 145.5 (Ar-4′-C), 147.3 (Ar-3′-C), 151.0 (Ar-1″-C–N). Anal. Calcd. for $C_{19}H_{22}N_2O_2$: C, 73.52; H, 7.14; N, 9.03. Found: C, 73.77; H, 7.16; N. 8.99.

*4.1.7.4. 1-(3-(Benzo[d] [1,3]dioxol-5-yl)propyl)-4-phenylpiperazine (LASSBio-632, **7e**).* Prepared from nucleophilic substitution of mesylate (**20A**) with *N*-phenylpiperazine, as a yellow oil; 0.158 g, (98%); Rf = 0.46 ($CHCl_3$:EtOH 5%); IR (film, cm^{-1}) 3036, 2942, 2818, 1600, 1503, 1489, 1444, 1379, 1244, 1187, 1139, 1038; ^1H NMR ($CDCl_3$): δ 1.73–1.88 (m, 2H, $ArCH_2CH_2CH_2N$) 2.36–2.44 (m, 2H, $ArCH_2CH_2CH_2N$), 2.54–2.62 (m, 6H, $ArCH_2CH_2CH_2N$ (2H) e NCH_2CH_2NAr (4H)), 3.17–3.22 (m, 4H, NCH_2CH_2NAr), 5.90 (s, 2H, OCH_2O), 6.63 (dd, J^3 = 7.8 Hz, J^4 = 1.7 Hz, 1H, Ar– H-6′), 6.69 (d, J^4 = 1.7 Hz, 1H, Ar–H-2′), 6.72 (d, J^3 = 7.8 Hz, 1H, Ar–H-5′), 6.80–6.94 (m, 3H, N–Ar–H-2″, 4″e 6″), 7.20–7.31 (m, 2H, N–Ar–H-3″ e 5″);^{13}C NMR ($CDCl_3$): δ 28,5 ($ArCH_2CH_2CH_2N$), 33,1 ($ArCH_2CH_2CH_2N$), 48,8 (NCH_2CH_2NAr), 53,1 (NCH_2CH_2NAr), 57,5 ($ArCH_2CH_2CH_2N$), 100,4 (OCH_2O), 107,8 (Ar-2′-CH), 108,6 (Ar-5′-CH), 115,7 (Ar-2″ e 6″- 2CH), 119,5 (Ar-6′-CH), 120,7 (Ar-4″-CH), 128,8 (Ar-3″ e 5″-2CH), 135,6 (Ar-1′-C), 145,3 (Ar-4′-C), 147,3 (Ar-3′-C), 151,0 (Ar-1″-C–N). Anal. Calcd. for $C_{20}H_{24}N_2O_2$: C, 74.04; H, 7.46; N, 8.64. Found: C, 73.78; H, 7.44; N. 8.65.

4.1.7.5. 1-(4-(Benzo[d] [1,3]dioxol-5-yl)butyl)-4-phenylpiperazine (LASSBio-675, ***7f****).* Prepared from nucleophilic substitution of mesylate (**24A**) with *N*-phenylpiperazine, as a beige solid; 0.142 g (93%); Rf = 0.47 ($CHCl_3$:EtOH 5%), mp 53–54 °C; IR (KBr, cm^{-1}) 3011, 2933, 2822, 1600, 1578, 1505, 1454, 1324, 1238, 1153, 1038; ^1H NMR ($CDCl_3$): δ 1.56–1.59 (m, 4H, $ArCH_2CH_2CH_2CH_2N$) 2.36–2.43 (m, 2H, $ArCH_2CH_2CH_2CH_2N$), 2.53–2.60 (m, 6H, $ArCH_2CH_2CH_2CH_2N$ (2H) e NCH_2CH_2NAr (4H)), 3.17–3.22 (m, 4H, NCH_2CH_2NAr), 5.90 (s, 2H, OCH_2O), 6.62 (d, $J^3 = 7.9$ Hz, 1H, Ar–H-6′), 6.67 (s, 1H, Ar–H-2′), 6.72 (d, $J^3 = 7.9$ Hz, 1H, Ar–H-5′), 6.80–6.96 (m, 3H, Ar–H-2″, 4″ e 6″), 7.22–7.33 (m, 2H, N–Ar–H-3″ e 5″); ^{13}C NMR ($CDCl_3$): δ 26.1 ($ArCH_2CH_2CH_2CH_2N$) 29.4 ($ArCH_2CH_2CH_2CH_2N$), 35.3 ($ArCH_2CH_2CH_2CH_2N$), 48.9 (NCH_2CH_2NAr), 53.1 (NCH_2CH_2NAr), 58.3 ($ArCH_2CH_2CH_2CH_2N$), 100.5 (OCH_2O), 107.8 (Ar-2′-CH), 108.6 (Ar-5′-CH), 115.8 (Ar-2″ e 6″- 2CH), 119.4 (Ar-6′-CH), 120.7 (Ar-4″-CH), 128.8 (Ar-3″ e 5″-2CH), 136.0 (Ar-1′-C), 145.3 (Ar-4′-C), 147.3 (Ar-3′-C), 151.1 (Ar-1″-C–N). Anal. Calcd. for $C_{21}H_{26}N_2O_2$: C, 74.52; H, 7.74; N, 8.28. Found: C, 74.71; H, 7.77; N. 8.26.

4.1.7.6. 1-(2-(Benzo[d] [1,3]dioxol-5-yl)ethyl)-4-(2-methoxyphenyl) piperazine (LASSBio-772, ***7g****).* Prepared from nucleophilic substitution of mesylate (**18A**) with *N*-(2-methoxyphenyl)piperazine, as a white solid; 0.167 g (98%); Rf = 0.55 ($CHCl_3$:EtOH 5%), mp 90–92 °C; IR (KBr, cm^{-1}) 3022, 2951, 2804, 1586, 1494, 1459, 1445, 1314, 1245, 1135, 1038, 752; ^1H NMR ($CDCl_3$): δ 2.59–2.83 (m, 8H, $ArCH_2CH_2N$ (4H) e NCH_2CH_2NAr (4H)); 3.11–3.16 (m, 4H, NCH_2CH_2NAr); 3.75 (s, 3H, OCH_3); 5.91 (s, 2H, OCH_2O); 6.65–6.69 (m, 1H, Ar– H-6′); 6.73 (m, 1H, Ar–H-2′); 6.75 (d, $J^3 = 7.7$ Hz, 1H, Ar–H-5′); 6.85–6.88 (m, 1H, N–Ar–H-6″); 6.91–7.05 (m, 3H, N–Ar–H-3″, 4″ e 5″); ^{13}C NMR ($CDCl_3$): δ 33.1 ($ArCH_2CH_2N$); 50.4 (NCH_2CH_2Ar); 53.1 (NCH_2CH_2NAr); 55.2 (Ar-4″-C–OCH_3); 60.6 ($ArCH_2CH_2N$); 100.6 (OCH_2O); 108.0 (Ar-2′-CH); 109.0 (Ar-5′-CH); 111.0 (Ar-3″, 1CH); 118.0 (Ar-6″, 1CH); 120.8 (Ar-5″, 1CH); 121.2 (Ar-6′, 1CH); 122.7 (Ar-4″- 1CH); 133.9 (Ar-1′-C); 141.1 (Ar-1″-C–N); 145.6 (Ar-4′-C); 147.3 (Ar-3′-C); 152.1 (Ar-2″-C–OCH_3). Anal. Calcd. for $C_{20}H_{24}N_2O_3$: C, 70.56; H, 7.11; N, 8.23. Found: C, 70.79; H, 7.13; N. 8.21.

4.1.7.7. 1-(3-(Benzo[d] [1,3]dioxol-5-yl)propyl)-4-(2-methoxyphenyl) piperazine (LASSBio-773, ***7h****).* Prepared from nucleophilic substitution of mesylate (**20A**) with *N*-(2-methoxyphenyl)piperazine, as a light-yellow oil; 0.172 g (97%); Rf = 0.41 ($CHCl_3$:EtOH 5%); IR (Film, cm^{-1}): 3061, 2940, 2814, 1594, 1501, 1489, 1444, 1242, 1183, 1136, 1037, 749; ^1H NMR ($CDCl_3$): δ 1.75–1.90 (m, 2H, $ArCH_2CH_2CH_2N$) 2.41–2.48 (m, 2H, $ArCH_2CH_2CH_2N$); 2.55–2.63 (m, 2H, $ArCH_2CH_2CH_2N$) 2.64–2.69 (m, 4H, NCH_2CH_2NAr); 3.10–3.14 (m, 4H, NCH_2CH_2NAr); 3.75 (s, 3H, OCH_3); 5.91 (s, 2H, OCH_2O); 6.64 (d, $J^3 = 7.8$ Hz, $J^4 = 1.6$ Hz, 1H, Ar– H-6′); 6.70 (m, 1H, Ar–H-2′); 6.73 (d, $J^3 = 7.8$ Hz, 1H, Ar–H-5′); 6.84–6.87 (m, 1H, N–Ar–H-6″); 6.90–7.04 (m, 3H, N–Ar–H-3″, 4″ e 5″); ^{13}C NMR ($CDCl_3$): δ 28.5 ($ArCH_2CH_2CH_2N$); 33.2 ($ArCH_2CH_2CH_2N$); 50.3 (NCH_2CH_2NAr); 53.2 (NCH_2CH_2NAr); 55.1 (Ar-4″-C-OCH_3); 57.6 ($ArCH_2CH_2CH_2N$); 100.5 (OCH_2O); 107.8 (Ar-2′-CH); 108.6 (Ar-5′-CH); 111.0 (Ar-3″, 1CH); 118.0 (Ar-6″-1CH); 120.8 (Ar-6′, Ar-5″-2CH); 122.6 (Ar-4″- 1CH); 135.7 (Ar-1′-C); 141.1 (Ar-1″-C–N); 145.6 (Ar-4′-C); 147.3 (Ar-3′-C); 152.0 (Ar-4″-C-OCH_3). Anal. Calcd. for $C_{21}H_{26}N_2O_3$: C, 71.16; H, 7.39; N, 7.90. Found: C, 70.98; H, 7.36; N. 7.92.

4.1.7.8. 1-(2-(Benzo[d] [1,3]dioxol-5-yl)ethyl)-4-(4-fluorophenyl) piperazine (LASSBio-681, ***7i****).* Prepared from nucleophilic substitution of mesylate (**18A**) with *N*-(4-fluorophenyl)piperazine, as a white solid; 0.148 g (90%); Rf = 0.48 ($CHCl_3$:EtOH 5%), mp 89–90 °C; IR (KBr, cm^{-1}) 3053, 2931, 2822, 1605, 1509, 1499, 1489, 1317, 1240, 1147, 1042, 813; ^1H NMR ($CDCl_3$): δ 2.58–2.82 (m, 8H, $ArCH_2CH_2N$ (4H) e NCH_2CH_2NAr (4H)) 3.14–3.18 (m, 4H, NCH_2CH_2NAr); 5.88 (s, 2H, OCH_2O); 6.64–6.69 (m, 1H, Ar– H-6′); 6.72–6.76 (m, 2H, Ar–H-2′ e 5′); 6.84–6.91 (m, 2H, N–Ar–H-2″ e 6″); 6.93–7.01 (m, 2H, N–Ar–H-3″ e 5″); ^{13}C NMR ($CDCl_3$): δ 33.1 ($ArCH_2CH_2N$); 49.9 (NCH_2CH_2NAr); 53.0 (NCH_2CH_2NAr); 60.4 ($ArCH_2CH_2N$); 100.6 (OCH_2O); 108.0 (Ar-2′-CH); 109.0 (Ar-5′-CH); 115.4 (d, $J^2 = 22.3$ Hz, Ar-3″ e 5″-2CH); 117.7 (d, $J^3 = 7.75$ Hz, Ar-2″ e 6″- 2CH); 121.3 (Ar-6′-CH); 133.8 (Ar-1′-C); 145.7 (Ar-4′-C); 147.4 (Ar-3′-C); 147.8 (Ar-1″-C–N); 157.0 (d, $J^1 = 237.5$ Hz, Ar-4″-C–F). Anal. Calcd. for $C_{19}H_{21}N_2O_2F$: C, 69.49; H, 6.45; N, 8.53. Found: C, 69.70; H, 6.53; N. 8.55.

4.1.7.9. 1-(2-(Benzo[d] [1,3]dioxol-5-yl)ethyl)-4-(4-chlorophenyl) piperazine (LASSBio-682, ***7j****).* Prepared from nucleophilic substitution of mesylate (**18A**) with *N*-(4-chlorophenyl)piperazine, as a white solid; 0.157 g (91%); Rf = 0.50 ($CHCl_3$:EtOH 5%), mp 94–95 °C; IR (KBr, cm^{-1}) 2956, 2822, 1596, 1487, 1440, 1356, 1239, 1187, 1134, 1040, 822; ^1H NMR ($CDCl_3$): δ 2.55–2.79 (m, 8H, $ArCH_2CH_2N$ (4H) e NCH_2CH_2NAr (4H)) 3.15–3.20 (m, 4H, NCH_2CH_2NAr); 5.91 (s, 2H, OCH_2O); 6.65 (dd, $J^3 = 7.8$ Hz, $J^4 = 1.6$ Hz, 1H, Ar– H-6′); 6.70–6.76 (m, 2H, Ar–H-2′ e 5′); 6.79–6.86 (m, 2H, N–Ar–H-2″, e 6″); 7.15–7.22 (m, 2H, N–Ar–H-3″ e 5″); ^{13}C NMR ($CDCl_3$): δ 33.1 ($ArCH_2CH_2N$); 48.9 (NCH_2CH_2NAr); 52.9 (NCH_2CH_2NAr); 60.4 ($ArCH_2CH_2N$); 100.6 (OCH_2O); 108.0 (Ar-2′-CH); 108.7 (Ar-5′-CH); 117.0 (Ar-2″ e 6″- 2CH); 121.2 (Ar-6′-CH); 124.2 (Ar-4″-C–Cl); 128.7 (Ar-3″ e 5″-2CH); 133.7 (Ar-1′-C); 145.6 (Ar-4′-C); 147.4 (Ar-3′-C); 149.7 (Ar-1″-C–N). Anal. Calcd. for $C_{19}H_{21}N_2O_2Cl$: C, 66.18; H, 6.14; N, 8.12. Found: C, 65.99; H, 6.15; N. 8.14.

4.1.7.10. 1-(2-(Benzo[d] [1,3]dioxol-5-yl)ethyl)-4-(4-methoxyphenyl) piperazine (LASSBio-683, ***7k****).* Prepared from nucleophilic substitution with mesylate (**18A**) by *N*-(4-methoxyphenyl)piperazine, as a white solid; 0.150 g (88%); Rf = 0.44 ($CHCl_3$:EtOH 5%), mp 112–113 °C; IR (KBr, cm^{-1}) 2922, 2807, 1512, 1444, 1247, 1238, 1185, 1130, 1035, 824; ^1H NMR ($CDCl_3$): δ 2.58–2.83 (m, 8H, $ArCH_2CH_2N$ (4H) e NCH_2CH_2NAr (4H)) 3.12–3.16 (m, 4H, NCH_2CH_2NAr); 3.75 (s, 3H, OCH_3); 5.91 (s, 2H, OCH_2O); 6.65–6.69 (m, 1H, Ar– H-6′); 6.73–6.76 (m, 2H, Ar–H-2′ e Ar–H-5′); 6.82–6.87 (m, 2H, N–Ar–H-2″, e 6″); 6.89–6.95 (m, 2H, N–Ar–H-3″ e 5″); ^{13}C NMR ($CDCl_3$): δ 33.1 ($ArCH_2CH_2N$); 50.4 (NCH_2CH_2NAr); 53.1 (NCH_2CH_2NAr); 55.3 (Ar-4″-C-OCH_3); 60.4 ($ArCH_2CH_2N$); 100.6 (OCH_2O); 108.0 (Ar-2′-CH); 108.9 (Ar-5′-CH); 114.2 (Ar-3″ e 5″-2CH); 117.9 (Ar-2″ e 6″- 2CH); 121.2 (Ar-6′-CH); 133.8 (Ar-1′-C); 145.5 (Ar-4′-C e Ar-1″-C–N); 147.3 (Ar-3′-C); 153.6 (Ar-4″-C-OCH_3). Anal. Calcd. for $C_{20}H_{24}N_2O_3$: C, 70.56; H, 7.11; N, 8.23. Found: C, 70.38; H, 7.10; N. 8.24.

4.1.7.11. 1-(2-Phenoxyethyl)-4-phenylpiperazine (LASSBio-728, ***8a****).* Prepared from nucleophilic substitution of mesylate (**13B**) with *N*-phenylpiperazine, as a white solid; 0.137 g (97%); Rf = 0.58 ($CHCl_3$:EtOH 5%); mp 79–81 °C; IR (KBr, cm^{-1}): 3056, 2926, 2828, 1598, 1494, 1455, 1318, 1190, 1049; ^1H NMR ($CDCl_3$): δ 2.71–2.76 (m, 4H, NCH_2CH_2NAr), 2.86 (t, $J = 5.8$ Hz, 2H, OCH_2CH_2N), 3.19–3.24 (m, 4H, NCH_2CH_2NAr), 4.14 (t, $J = 5.8$ Hz, 2H, OCH_2CH_2N), 6.81–6.97 (m, 6 H, Ar–H-2′, H-6′, H-4′, H-2″, H-6″ e H-4″), 7.22–7.32 (m, 4H, Ar–H-3′,H-5′,H-3″ e H-5″); ^{13}C NMR ($CDCl_3$): δ 48.9 (NCH_2CH_2NAr), 53.5 (OCH_2CH_2N), 57.1 (NCH_2CH_2NAr), 65.7 (OCH_2CH_2N), 114.4 (Ar-2′ e 6′, 2CH), 115.9 (Ar-2″ e 6″, 2CH), 119.5 (Ar-4″-C), 120.6 (Ar-4′-C), 128.9 (Ar-3″ e 5″, 2CH), 129.3 (Ar-3′ e 5′, 2CH), 151.1 (Ar-1″-C–N), 158.5 (Ar-1′-C-O). Anal. Calcd. for $C_{18}H_{22}N_2O$: C, 76.56; H, 7.85; N, 9.92. Found: C, 76.69; H, 7.82; N. 9.95.

4.1.7.12. 1-(3-Phenoxypropyl)-4-phenylpiperazine (LASSBio-729, ***8b****).* Prepared from nucleophilic substitution of mesylate (**16B**) with *N*-phenylpiperazine, as a yellow oil; 0.107 g (92%); Rf = 0.54

($CHCl_3$:EtOH 5%); IR (Film, cm^{-1}): 3037, 2949, 2818, 1599, 1587, 1496, 1243, 1180, 1035; ^1H NMR ($CDCl_3$): δ 2.00 (qi, J = 6.3 Hz, 2H, $OCH_2CH_2CH_2N$), 2.53–2.64 (m, 6H, $OCH_2CH_2CH_2N$ (2H) e NCH_2CH_2NAr (4H)), 3.17–3.22 (m, 4H, NCH_2CH_2NAr), 4.02 (t, J = 6.3 Hz, 2H, $OCH_2CH_2CH_2N$), 6.80–6.96 (m, 6 H, Ar–H-2′, H-6′, H-4′, H-2″, H-6″ e H-4″), 7.20–7.31 (m, 4H, Ar–H-3′,H-5′,H-3″ e H-5″); ^{13}C NMR ($CDCl_3$): δ 26.6 ($OCH_2CH_2CH_2N$) 48.9 (NCH_2CH_2NAr), 53.1 (NCH_2CH_2NAr), 55.0 ($OCH_2CH_2CH_2N$), 65.8 ($OCH_2CH_2CH_2N$), 114.3 (Ar-2′ e 6′, 2CH), 115.8 (Ar-2″ e 6″, 2CH), 119.4 (Ar-4″-C), 120.4 (Ar-4′-C), 128.9 (Ar-3″ e 5″, 2CH), 129.2 (Ar-3′ e 5′, 2CH), 151.1 (Ar-1″-C–N), 158.8 (Ar-1′-C-O). Anal. Calcd. for $C_{19}H_{24}N_2O$: C, 76.99; H, 8.16; N, 9.45. Found: C, 77.18; H, 8.13; N. 9.46.

4.1.7.13. 1-Phenethyl-4-phenylpiperazine (LASSBio-771, ***8d****).* Prepared from nucleophilic substitution of mesylate (**18B**) with *N*-phenylpiperazine, as a white solid; 0.340 g (91%); Rf = 0.50 ($CHCl_3$:EtOH 5%); mp 70–72 °C; IR (KBr, cm^{-1}) 3024, 2945, 2827, 1600, 1576, 1495, 1445, 1312, 1140; ^1H NMR ($CDCl_3$): δ 2.64–2.70 (m, 6H, $ArCH_2CH_2N$ (2H) e NCH_2CH_2NAr (4H)) 2.77–2.89 m, 2H, $ArCH_2CH_2N$ (2H), 3.20–3.25 (m, 4H, NCH_2CH_2NAr), 6.81–6.95 (m, 3H, H-2″, H-4″, H-6″), 7.15–7.33 (m, 7H, H-3″, H-5″, H-2′ H-3′, H-4′, H-5′, H-6′); ^{13}C NMR ($CDCl_3$): δ 33.4 ($ArCH_2CH_2N$), 49.0 (NCH_2CH_2NAr), 53.0 (NCH_2CH_2NAr), 60.3 ($ArCH_2CH_2N$), 115.8 (Ar-2″ e 6″, 2CH), 119.5 (Ar-4″-C), 125.9 (Ar-4′-C), 128.2 (Ar-3′ e 5′, 2CH), 128.5 (2′ e 6′, 2CH), 128.9 (Ar-3″ e 5″, 2CH), 140.0 (Ar-1′-C–C), 151.1 (Ar-1″-C–N). Anal. Calcd. for $C_{18}H_{22}N_2$: C, 81.16; H, 8.32; N, 10.52. Found: C, 80.98; H, 8.35; N. 10.52.

4.1.7.14. 1-Phenyl-4-(3-phenylpropyl)piperazine (LASSBio-724, ***8e****).* Prepared from nucleophilic substitution of mesylate (**20B**) with *N*-phenylpiperazine, as a yellow oil; 0.127 g (90%); Rf = 0.54 ($CHCl_3$:EtOH 5%); IR (Film, cm^{-1}) 3025, 2942, 2816, 1600, 1578, 1496, 1453, 1311, 1140; ^1H NMR ($CDCl_3$): δ 1.80–1.96 (m, 2H, $ArCH_2CH_2CH_2N$) 2.41–2.49 (m, 2H, $ArCH_2CH_2CH_2N$), 2.59–2.72 (m, 6H, $ArCH_2CH_2CH_2N$ (2H) e NCH_2CH_2NAr, (4H)), 3.20–3.25 (m, 4H, NCH_2CH_2NAr), 6.82–6.98 (m, 3H, H-2″, H-4″, H-6″), 7.16–7.35 (m, 7H, H-3″, H-5″, H-2′, H-3′, H-4′, H-5′, H-6′); ^{13}C NMR ($CDCl_3$): δ 28.4 ($ArCH_2CH_2CH_2N$), 33.5 ($ArCH_2CH_2CH_2N$), 48.9 (NCH_2CH_2NAr), 53.0 (NCH_2CH_2NAr), 57.8 ($ArCH_2CH_2CH_2N$), 115.8 (Ar-2″ e 6″, 2CH), 119.4 (Ar-4″-C), 125.6 (Ar-4′-C), 128.1 (Ar-3′ e 5′, 2CH), 128.2 (2′ e 6′, 2CH), 128.8 (Ar-3″ e 5″, 2CH), 141.9 (Ar-1′-C–C),151.1 (Ar-1″-C–N). Anal. Calcd. for $C_{19}H_{24}N_2$: C, 81.38; H, 8.63; N, 9.99. Found: C, 81.68; H, 8.60; N. 10.02.

4.1.7.15. 1-Phenyl-4-(4-phenylbutyl)piperazine (LASSBio-730, ***8f****).* Prepared from nucleophilic substitution of mesylate (**24B**) with *N*-phenylpiperazine, as a beige solid; 0.122 g (92%); Rf = 0.50 ($CHCl_3$:EtOH 5%), mp 47–48 °C; IR (KBr, cm^{-1}) 3026, 2927, 2817, 1601, 1575, 1505, 1496, 1315, 1035; ^1H NMR ($CDCl_3$): δ 1.48–1.75 (m, 4H, $ArCH_2CH_2CH_2CH_2N$), 2.37–2.45 (m, 2H, $ArCH_2CH_2CH_2CH_2N$), 2.56–2.68 (m, 6H, $ArCH_2CH_2CH_2CH_2N$ (2H) e NCH_2CH_2NAr (4H)), 3.17–3.22 (m, 4H, NCH_2CH_2NAr), 6.81–6.95 (m, 3H, H-2″, H-4″, H-6″), 7.13–7.32 (m, 7H, H-3″, H-5″, H-2′ H-3′, H-4′, H-5′, H-6′); ^{13}C NMR ($CDCl_3$): δ 26.3 ($ArCH_2CH_2CH_2CH_2Ar$), 29.2 ($ArCH_2CH_2CH_2CH_2N$), 35.6 ($ArCH_2CH_2CH_2CH_2N$), 48.9 (NCH_2CH_2NAr), 53.1 (NCH_2CH_2NAr), 58.3 ($ArCH_2CH_2CH_2CH_2N$), 115.8 (Ar-2″ e 6″, 2CH), 119.4 (Ar-4″-C), 125.5 (Ar-4′-C), 128.1 (Ar-3′ e 5′, 2CH), 128.2 (2′ e 6′, 2CH), 128.9 (Ar-3″ e 5″, 2CH), 142.2 (Ar-1′-C–C),151.1 (Ar-1″-C–N). Anal. Calcd. for $C_{20}H_{26}N_2$: C, 81.59; H, 8.90; N, 9.51. Found: C, 81.71; H, 8.88; N. 9.49.

*4.1.8. General procedure for preparation of N-phenylpiperazine derivatives (**7c**) and (**8c**) through reductive amination*

A mixture of the corresponding aromatic aldehydes (**25A**) or (**25B**) (0.70 mmol), *N*-phenylpiperazine (0.11 mL, 0.72 mmol) and 0.02 g of montmorillonite K10 clay was underwent to microwave radiation in a domestic oven during 4 min at 20% of maximum potency (180 MHz). At the end of this time, *N*-phenylpiperazine (0.11 mL, 0.72 mmol) was added and the procedure repeated. After that, the mixture was underwent to microwave radiation considering 100% of potency (900 MHz) during 3 min (3x). After cooling to room temperature, a solution of 0.117 g (3.00 mmol) of sodium borohydride in methanol (10 mL) was added and the mixture was vigorously stirred at room temperature for 30 min. Then, the mixture was extracted with ethyl acetate (3 × 20 mL) and the combined organic extracts were successively washed with saturated aqueous Na_2CO_3 solution (2 × 10 mL), brine (10 mL) and dried over anhydrous sodium sulfate and evaporated under reduced pressure. The obtained residue was separated by chromatography on silica gel eluted with dichloromethane, followed by chloroform to give the corresponding *N*-phenylpiperazine derivatives (**7c**) and (**8c**), as described next.

4.1.8.1. 1-((Benzo[d] [1,3]dioxol-5-yl)methyl)-4-phenylpiperazine (LASSBio-719A, ***7c****).* White solid; 0.106 g (90%); Rf = 0.47 ($CHCl_3$:EtOH 5%); mp 83–85 °C; IR (KBr, cm^{-1}) 3016, 2955, 2821, 1598, 1491, 1438, 1333, 1240, 1040; ^1H NMR ($CDCl_3$): δ 2.54–2.59 (m, 4H, NCH_2CH_2NAr), 3.15–3.20 (m, 4H, NCH_2CH_2NAr), 3.45 (s, 2H, $ArCH_2N$), 5.92 (s, 2H, OCH_2O), 6.75–6.92 (m, 6H, Ar–H-2′, H-5′, H-6′, N–Ar–H-2″, 4″ e 6″), 7.20–7.28 (m, 2H, N–Ar–H-3″ e 5″); ^{13}C NMR ($CDCl_3$): δ 48.9 (NCH_2CH_2NAr), 52.8 (NCH_2CH_2NAr), 62.6 ($ArCH_2N$), 100.7 (OCH_2O), 107.9 (Ar-2′-CH), 109.3 (Ar-5′-CH), 115.8 (Ar-2″ e 6″- 2CH), 119.4 (Ar-4″-CH), 122.1 (Ar-6′-CH), 128.9 (Ar-3″ e 5″-2CH), 131.7 (Ar-1′-C), 146.4 (Ar-4′-C), 147.5 (Ar-3′-C), 151.2 (Ar-1″-C–N). Anal. Calcd. for $C_{18}H_{20}N_2O_2$: C, 72.95; H, 6.80; N, 9.45. Found: C, 72.80; H, 6.78; N. 9.47.

4.1.8.2. 1-Benzyl-4-phenylpiperazine (LASSBio719B, ***8c****).* Colorless Oil; 0.173 g (98%); Rf = 0.54 ($CHCl_3$:EtOH 5%); IR (Film, cm^{-1}) 3026, 2939, 2815, 1600, 1502, 1495, 1453, 1335, 1228, 1145; ^1H NMR ($CDCl_3$): δ 2.58–2.63 (m, 4H, NCH_2CH_2NAr), 3.17–3.22 (m, 4H, NCH_2CH_2NAr), 3.56 (s, 2H, $ArCH_2N$), 6.80–6.93 (m, 3H, N–Ar–H-2″, 4″ e 6″), 7.20–7.38 (m, 7H, Ar–H-2′, H-3′, H-4′, H-5′, H-6′, N–Ar-H-3″ e 5″); ^{13}C NMR ($CDCl_3$): δ 48.8 (NCH_2CH_2NAr), 52.8 (NCH_2CH_2NAr), 62.8 ($ArCH_2N$), 115.7 (Ar-2′ e 6′, 2CH), 119.3 (Ar-4′-C), 126.9 (Ar-4″-C), 128.0 (Ar-2″ e 6″, 2CH), 128.8 (3″ e 5″, 2CH), 128.9 (Ar-3′ e 5′, 2CH), 137.6 (Ar-1′-C-C), 151.0 (Ar-1″-C–N). Anal. Calcd. for $C_{17}H_{20}N_2$: C, 80.91; H, 7.99; N, 11.10. Found: C, 81.12; H, 8.01; N. 11.08.

4.2. Pharmacological evaluation

All animal procedures were approved by the institutional ethical committee (CEUA-CCS/UFRJ).

4.2.1. Measurement of contractile responses

4.2.1.1. Rabbit aorta. Rabbit aortic rings (3–4 mm wide) were fixed in an organ bath chamber filled with physiological solution under a resting tension of 20 mN, and left to equilibrate for 30 min during which time the physiological solution was changed twice. Following, phenylephrine cumulative concentration–response curves ($5 \times 10^{-9} - 5 \times 10^{-4}$ M) were performed in the absence or presence of 30 μM *N*-phenylpiperazine derivatives (**7a**–**k**), (**8a**–**f**) or BMY7378 (**6**), pre-incubated for 30 min. The phenylephrine log EC_{50} values were estimated independently for each experiment by non-linear regression analysis using the software GraphPad Prism 4.0 (sigmoidal dose-response equation). The EC_{50} ratio (concentration ratio, r) was obtained from the phenylephrine EC_{50} value observed in the presence of a compound divided by the value of the corresponding control curve, and used for estimation of the equilibrium dissociation constant of the antagonist (K_B) through the Schild equation: $r - 1 = [B]/K_B$, where [B] refers to the antagonist concentration [41].

4.2.1.2. Rat aorta. Rat aortic rings (3 mm wide) were fixed in an organ bath chamber filled with physiological solution under a resting tension of 20 mN, and left to equilibrate for 60 min during which time the physiological solution was changed twice. The developed active tension was measured isometrically using a Grass Transducer (FT-03). Data were acquired and analyzed using Chart 3.4.9 software (MacLab, U.S.A). Tissues were precontracted with 1 μM noradrenaline and at the plateau of the tonic contraction acetylcholine (1 μM) was added to access the endothelium-dependent relaxation. Rings that relaxed at least 50% were considered as endothelium-intact and used in the experiments [59]. Alternatively, the endothelium was mechanically removed and experiments were performed in the continuous presence of L-NAME 100 μM (an inhibitor of nitric oxide synthase). Following, the rings were washed, left to equilibrate for 1 h and then a cumulative concentration–response curve was performed in the before (control) or after the addition of LASSBio-772 (**7g**) (0.1, 0.3 and 1 nM), prazosin (1–10 nM) or BMY7378 (10 nM) 1 h before. The EC_{50} values were estimated independently for each experiment by non-linear regression using the software GraphPad Prism 4.0 (San Diego - California, USA, www.graphpad.com). The EC_{50} values observed in the presence of a molar concentration of the antagonists were divided by the values of the control curve to obtain the concentration ratios (r). Then, we performed a Schild regression for each antagonist using the equation $\log(r-1) = \log[B] - \log K_B$, where K_B represents the dissociation constant of the antagonist and [B] represents the concentration of the antagonist. This equation describes a linear relation between $\log(r-1)$ and [B] that intercepts the ordinate at a value corresponding to the pA_2, an empirical parameter corresponding to the antagonist concentration at which r would be 2, and classically used to estimate antagonist potency since it is equal to K_B in ideal conditions like in our experiments [41,60]. As BMY7378 was used in a single concentration (10 nM) the K_B values were individually calculated using Schild equation.

4.2.2. Rat liver preparation and binding assays

Rat liver membranes were prepared according to Michel *et al* [61]. Briefly, rat livers were removed, placed in a cold solution (sucrose 0.25 M, TRIS 5 mM, EGTA 1 mM, pH 7.4) and minced. Following the tissue was homogenized in a Potter and the suspension was filtered through four layers of gauze. After this procedure the material was centrifuged (5000× *g*, 20 min, 4 °C) twice, and the pellet was ultracentrifuged at 80 000× *g* (20 min, 4 °C). The final pellet was resuspended in sucrose solution (sucrose 0.25 M, TRIS 5 mM, pH 7.4) and stored in liquid nitrogen. Rat salivary glands were obtained according to the method described previously by Michel et al. [62]. The protein content was determined according to Bradford [63].

In all competition assays, about 150 μg of protein were incubated for 45 min at 25 °C in a solution (500 μl) containing 0.1 (liver) or 0.06 nM (salivary gland) [^{3}H]-prazosin, TRIS-HCl 50 mM and EDTA 1 mM (pH 7.4) [61,62], in the absence or presence of LASSBio-772 (**7g**) (3×10^{-10} to 3×10^{-7} M) or prazosin (10^{-10} to 10^{-8} M). The incubation was finished by dilution (4 times) of the samples with 3 mL of ice-cold buffer (TRIS-HCl 50 mM pH 7,4), followed by rapid filtration under vacuum using a glass fiber filter (GMF 3, Filtrak, Germany). After drying, filters were added to a scintillation mixture [POPOP (0.1 g/l) and PPO (4.0 g/l) in toluene], and the radioactivity was measured in a Packard liquid scintillation counter with 45% efficiency. The specific binding was calculated by subtracting the value of non-specific binding (measured in the presence of prazosin 1 μM) from the total value. The values of IC_{50} were calculated independently for each experiment by non-linear regression using the software GraphPad Prism 4.0 (San Diego - California, USA, www.graphpad.com) considering the presence of one subtype of receptor. The K_i values were calculated using the Cheng and Prusoff equation.

4.2.3. In vivo assays: hemodynamic measurements

New Zealand albino rabbits of either sex (3.0 kg) were anesthetized with sodium pentobarbital (40 mg/kg) administered through the marginal vein of the ear; anesthesia was complemented by another i.v. injection of 5 mg/kg sodium pentobarbital before the control period. After induction of anesthesia, a tracheotomy was performed and the lungs were artificially ventilated with room air using an animal ventilator (Ugo Basile, Model 7025, Biological Research Apparatus, Varese, Italy). Neuromuscular blockade was induced with pancuronium bromide (1 mg/kg, i.v.). The right femoral vein was catheterized to permit i.v. injections. The arterial pressure was continuously monitored through a catheter placed in the abdominal aorta via the right femoral artery. Cardiac output was measured with an electromagnetic probe connected to a flowmeter (Skalar MDL 1401, Skalar Instruments, The Netherlands) placed in the left ventricle. The mean arterial pressure was measured and the systemic vascular resistance was calculated dividing mean arterial pressure (mmHg) by cardiac output (ml/min), multiplied by 80 to transform in dyn s/cm^5 [64].

4.2.3.1. Experimental protocol. After a stabilization period of 15–30 min (control period), dose-response curves were performed with phenylephrine (0.1–100 μg/kg, i.v.) in the absence or presence of LASSBio-772 (**7g**) (0.1–100 μg/kg, i.v.) and the cardiovascular parameters were continuously recorded during 30 min. Alternatively blood pressure was monitored after treatment with LASSBio-772 (**7g**) or prazosin (**1**) (100 μg/kg, i.v.)

Acknowledgments

The authors thank UCB (BR), CAPES (BR), CNPq (BR), FAPERJ (BR), PRONEX (BR) and INCT-INOFAR (BR, #573.564/2008-6) for financial support and fellowships. Orlando da Rocha Moreira (UFRJ, Brazil) for technical assistance. Barreiro, E.J.; Fraga, C.A.M.; Noël, F.; Silva, C.L.M. and Tibiriçá, E. are seniors CNPq fellowships (Brazil).

Appendix. Supplementary material

Supplementary data related to this article can be found online at doi: 10.1016/j.ejmech.2011.04.032.

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