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Methylation of imidazoline related compounds leads to loss of α_2-adrenoceptor affinity. Synthesis and biological evaluation of selective I_1 imidazoline receptor ligands

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ABSTRACT

Methylated analogues of imidazoline related compounds (IRC) were prepared; their abilities to bind I_1 imidazoline receptors (I_1Rs), I_2 imidazoline binding sites (I_2BS) and α_2-adrenoceptor subtypes (α_2ARs) were assessed. Methylation of the heterocyclic moiety of IRC resulted in a significant loss of α_2AR affinity. Amongst the selective ligands obtained, LNP 630 (**4**) constitutes the first highly selective I_1R agent showing hypotensive activity after intravenous administration.

1. Introduction

I_1 Imidazoline receptors (I_1Rs), a subclass of the imidazoline binding sites (IBS), are involved in the central hypotensive activity of clonidine (Fig. 1) and related compounds.[1] Four substances have been proposed as their endogenous ligand (agmatine, clonidine displacing substance, harmane and imidazoleacetic acid-ribotide)[2–5] and different signalling pathways have been shown to be associated with these receptors.[6–8] I_1Rs are found in both peripheral and central nervous system and exhibit high affinity for clonidine.[1]

The others IBS are I_2 imidazoline binding sites (I_2BS), that bind idazoxan with high affinity but not clonidine and have been shown to be a modulatory site of monoamine oxidase (MAO)[9], and I_3 imidazoline binding sites (I_3BS) that modulate insulin secretion in pancreatic beta-cells.[10]

Imidazoline related compounds (IRC) is the term used to gather all compounds that bind to IBS. They include aminoimidazolines (also called iminoimidazolidines), 2-alkyl(aryl)imidazolines, aminooxazolines, aminopyrrolines, guanidines and primary amines. The study of I_1Rs has long been restricted by the lack of selectivity of IRC for these receptors over α_2-adrenoceptors (α_2ARs) and I_2BS. These residual affinities for α_2ARs and I_2BS complicated the interpretation of the IRC-induced hypotensive effects and the molecular characterization of I_1Rs. The first compounds with some selectivity for I_1Rs over α_2ARs were rilmenidine (Fig. 1) and moxonidine.[11] However, these two drugs still bind to α_2ARs with rather high affinity. More recently, a few ligands were described with much greater selectivity for I_1Rs over α_2ARs: benazoline[7] and PMS 952,[12] or over α_2ARs and I_2BS: AGN 192403[13] and LNP 509 (Fig. 1).[14] The latter, a methylated pyrrolinic isostere of rilmenidine, has detectable affinity for neither α_2ARs nor I_2BS but nevertheless reduces blood pressure after central administration.[14] This hypotensive effect seems to involve G-protein inwardly rectifying potassium channels.[15]

Efforts to obtain more selective IBS ligands and to understand structure activity–relationships governing their affinities have led

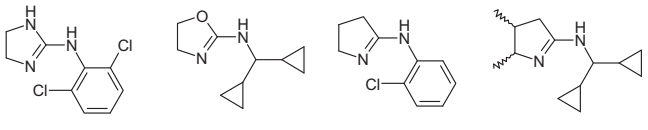

Figure 1. Structures of important imidazoline related compounds.

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to new series of IRC and models that can be used to predict requirements for high affinity and selectivity.[16–21]

Following the observation that the methylated LNP 509 showed a high selectivity profile, we decided to explore more in depths IRC heterocycle moieties and particularly their methylation on the carbon atom adjacent to the nitrogen. We studied this structural modification in three different chemical classes of IRC by synthesizing the methylated analogues of the three following reference compounds: clonidine for the aminoimidazoline family, rilmenidine for the aminooxazoline family and SD 067 (Fig. 1) for the aminopyrroline family. The latter compound is a pyrrolinic isostere of clonidine, synthesized in the 70s and showing similar hypotensive activity as clonidine itself.[22,23] SD 067 was of particular interest because its hypotensive effect after intravenous administration was preceded by a vasoconstriction weaker than that of clonidine. As this vasoconstriction is mainly due to a vascular α-adrenoceptor activation,[23] this compound seemed to be more selective for I_1Rs over α_2ARs than clonidine. This assumption is confirmed by binding studies in the present study.

The aim of this work was not only to obtain additional structure–activity relationship informations for IRC methylated on the heterocycle, but also to synthesize new tools selective for I_1Rs. These tools will be very useful for the characterization of I_1Rs, as the most widely used radiolabelled molecules to study I_1Rs ([^{3}H]clonidine and [^{125}I]paraiodoclonidine) still suffer from α_2AR nanomolar affinities.[24] Thanks to this methylation, we obtained the iodinated ligand LNP 911 (**10**) that shows unprecedented selectivity for I_1Rs over α_2ARs and I_2BS.[25]

These selective tools will also be useful for the study of centrally hypotensive agent mechanism of action and will provide prototypes for the development of novel therapeutic agents. Indeed, up to now, no selective IRC with hypotensive activity after systemic administration was described. LNP 630 (**4**), the methylated analogue of clonidine described in this study fulfils these criteria.

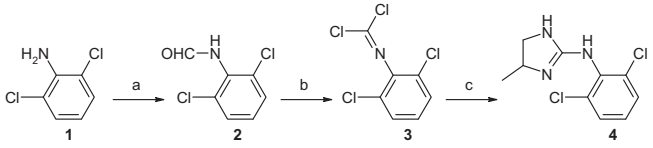
2. Synthesis and evaluation

2.1. Chemistry

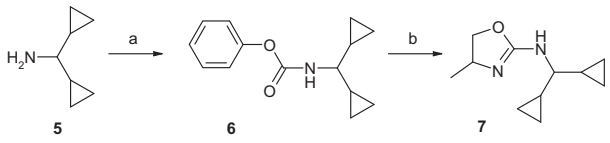
The methylated analogue **4** was obtained following the method described for the synthesis of [^{14}C]clonidine by Ehrhardt.[26] The dichloroimine **3** was synthesized from 2,6-dichloroaniline **1** by successive formylation, dehydration with $SOCl_2$ and chlorination with SO_2Cl_2 (Scheme 1). It was then reacted with commercially available 1,2-diaminopropane to form the imidazoline derivative **4**.

For compound **7**, we followed the strategy used for rilmenidine synthesis described in its original patent.[27] The dicyclopropylmethylamine **5** was first converted into the carbamate **6** by treatement with phenyl chloroformate (Scheme 2). Then, compound **6** was coupled to commercial 2-amino-1-propanol and cyclised to afford the oxazoline **7**.

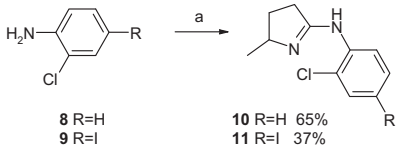
The aminopyrroline analogues were obtained by reaction of 5-methyl-2-pyrrolidinone with the corresponding anilines **8** and **9** (Scheme 3). This coupling was made in the presence of $POCl_3$ as dehydrating agent.[28]



Scheme 1. Compounds **1**–**4**. Reagents and conditions: (a) HCCOH, Ac_2O, 40 °C, 73%; (b) SO_2Cl_2, $SOCl_2$, 60 °C, 60%; (c) NH_2–$CH(CH_3)$–CH_2–NH_2, Et_2O, 75%.



Scheme 2. Compounds **5**–**7**. Reagents and conditions: (a) ClCOOPh, H_2O, Et_3N, 0 °C to room temperature, 92%; (b) NH_2-$CH(CH_3)$–CH_2–OH, H_2O, reflux, 61%; (c) (1) $SOCl_2$, $CHCl_3$, 0 °C, (2) H_2O, reflux, 8%.



Scheme 3. Compounds **8**–**11**. Reagents and conditions: (a) 5-methyl-2-pyrrolidinone, $POCl_3$, Cl-$(CH_2)_2$-Cl, 60 °C.

2.2. Pharmacology

2.2.1. Binding experiments

Affinities of compounds **4**, **7**, **10** and **11** for I_1Rs, I_2BS and the three subtypes of α_2ARs were determined by receptor binding assays. The radioligands used were [^{125}I]paraiodoclonidine, [^{3}H]idazoxan and [^{3}H]RX821002, respectively. Experiments were performed on PC 12 cells (I_1Rs), rabbit renal cortex (I_2BS) and membranes of Chinese Hamster Ovary (CHO) cells transfected with human α_2AR subtypes.

2.2.2. In vivo studies

Effects of compounds **4**, **7** and **10** on mean arterial blood pressure (MAP) of spontaneously hypertensive rats were determined after intravenous administrations (see Section 5 for details). We chose this route of administration to evaluate at the same time the hypotensive and the vasoconstrictive activities of the drugs.

3. Results and discussions

Binding characteristics of the four methylated compounds **4**, **7**, **10** and **11** as well as those of clonidine, rilmenidine and SD 067 were determined (Table 1).

The first observation is that compound SD 067 displays weaker affinities for the three α_2ARs than clonidine. This difference correlates with the weaker vasoconstrictive effect of SD 067 compared with clonidine after intravenous administration.[23] We had previously noticed that pyrrolinic isosteres of rilmenidine exhibited no detectable affinities for α_2ARs.[14] Taken together, these results suggest that the NH group of the imidazoline ring of clonidine may play the same role as the oxygen of the oxazoline ring of rilmenidine in the binding with α_2ARs.

The second observation is the effect of the methylations of clonidine, rilmenidine and SD 067 on the three α_2AR subtypes bindings. This structural modification dramatically reduces the affinities of **4** (displaying 224-, 42- and 617-fold weaker affinities compared with clonidine for the respective α_{2A}ARs, α_{2B}ARs and α_{2C}ARs) and of **7** (with 135-, 66- and more than 794-fold weaker affinities compared with rilmenidine). The same effect was observed with **10** showing no detectable affinities for the three α_2AR subtypes whereas SD 067 bounds weakly with pK_i of 5.52, 6.03 and 5.30 for the respective α_{2A}ARs, α_{2B}ARs and α_{2C}ARs. These results show the lack of steric tolerance on this heterocyclic position for the binding on α_2ARs within the three different chemical classes of IRC. Interestingly, Treder et al. recently reported that a larger addition on the same position of the imidazoline ring of IRC affected in a similar way their α_2AR affinities.[29]

Table 1
Binding data of references and compounds **4**, **7**, **10** and **11**

Compounds	pK_i ± sem				
	I_1Rs	I_2Bs	$\alpha2_A$ARs	$\alpha2_B$ARs	$\alpha2_C$ARs
Clonidine[a]	6.55 ± 0.04	<5	8.06 ± 0.11	7.50 ± 0.10	8.03 ± 0.12
4	9.03 ± 0.19	<5	5.71 ± 0.05	5.88 ± 0.08	5.24 ± 0.03
Rilmenidine[a]	7.95 ± 0.45	<5	7.44 ± 0.23	7.37 ± 0.22	7.90 ± 0.25
7	6.44 ± 0.03	<5	5.31 ± 0.11	5.55 ± 0.09	<5
SD067	7.11 ± 0.20	6.10 ± 0.09	5.52 ± 0.10	6.03 ± 0.12	5.30 ± 0.07
10	6.88 ± 0.21	<5	<5	<5	<5
11	9.66 ± 0.17	<5	5.59 ± 0.03	5.38 ± 0.02	<5

[a] Affinities for the three α_2-AR subtypes are taken from Ref. 31.

Our data also suggest that clonidine, rilmenidine and SD 067 share a common binding mode with α_2ARs. We can assume that the basic function of these three agents will take part in an ionic bond with the same amino acid residue(s) of α_2ARs. Moreover, these results are in agreement with those of Salminen et al.[30] showing that the CH_2 groups in the imidazoline ring of clonidine and others imidazoline related compounds pack against the seventh transmembrane helix of the α_{2A}-adrenoceptor. These authors obtained this result with docking simulations of ligands to an α_{2A}-adrenoceptor model based on the C_α-atom template of rhodopsin-like GPCRs. It can be easily assumed that addition of a methyl group on the heterocyclic positions of IRC will therefore disrupt the appropriate positioning of these ligands in the binding pocket of α_2ARs. This modification may prevent formation of an ionic bond with α_2ARs.

As I_1R affinity of these methylated analogues is concerned, different results were obtained according to their chemical classes. Firstly for the aminoimidazoline **4**, methylation increased I_1R affinity (pK_i = 6.55 for clonidine and 9.03 for **4**). This observation provides us with additional evidence that structural requirements for I_1Rs and α_2ARs are different.[14] Secondly for the aminopyrroline **10**, the addition of the methyl group had only a weak influence on I_1R affinity (pK_i = 7.11 for SD 067 and 6.88 for **10**). Finally for the aminooxazoline **7**, the modification of the heterocycle led to a decrease in I_1R affinity (pK_i = 7.95 for rilmenidine and 6.44 for **7**). These results point out differences in the structure–affinity relationships of the three IRC that may come from distinct I_1R binding modes.

Moreover, none of these methylated compounds exhibited significant affinity for I_2BS. It is not surprising for **4** and **7** since their unmethylated analogues do not bind to I_2BS, but for compound **10**, addition of the methyl helps reducing the I_2BS affinity. Therefore, this methylation appears to be a simple way to obtain ligands selective for I_1Rs over α_2ARs and I_2BS in both aminoimidazoline and aminopyrroline series.

Taken advantage of the improved selectivity of these methylated compounds, we further investigated new selective tools that can be radiochemically labelled. For that purpose, an iodine atom was added on the *para* position of compound **10** aromatic ring. Two reasons accounted for this choice. First, it has been shown that para iodination of clonidine increased significantly the I_1R affinity by a factor of 10–250.[7,31] The second reason is the high specific activity of iodine radioisotope ^{125}I (approximately 2000 Ci/mmol) that provides advantage for binding studies with low density receptors like I_1Rs (52 fmol/mg prot. in bovine rostral ventrolateral medullary membranes, 20 fmol/mg prot. in PC 12 cells, 44 fmol/mg prot. in bovine adrenal medullary membranes and 60 fmol/mg prot. in human platelets).[1,7,32–34] As expected, this iodinated compound **11** showed a significant increase in I_1R affinity (pK_i = 9.66 for **11** and 6.88 for **10**) together with a slight increase in affinities for some α_2ARs (see Table 1). Aminopyrroline **11** constitutes a pharmacological tool with unprecedented high affinity for I_1Rs and excellent selectivity profile over α_2ARs (11 749-, 19 055- and >45 709-fold over α_{2A}ARs, α_{2B}ARs and α_{2C}ARs, respectively) and I_2BS (>45 709-fold). Characterization and uses of the corresponding radiolabelled **11** has been previously described.[25] LNP 906, the azido derivative of **11** was also synthesized and characterized.[35] This photoaffinity ligand will be of great help for the identification of the molecular entity binding IRC.

The cardiovascular evaluation of the three methylated analogues **4**, **7** and **10** was then performed. For this purpose, effects on blood pressure (BP) following intravenous administration were studied in the spontaneous hypertensive rat (SHR) at the dose of 1 mg/kg (Fig. 2).

None of the three methylated analogues showed any vasoconstrictive effect at this dose whereas their corresponding unmethylated analogues are known to be vasoconstrictive in the same experimental conditions.[36] Therefore, methylation of that heterocyclic position is sufficient to abolish the vasoconstrictive component of the three IRC, corroborating in an in vivo system the α_2AR loss of affinity observed in vitro.

Concerning the hypotensive activity, only **4** was able to reduce BP at 1 mg/kg (79 ± 42 mm Hg vs 174 ± 26 mm Hg i.e., 45% in 5 min) when injected intravenously. Compound **4** represents therefore the first compound selective for I_1Rs over α_2ARs and I_2BS, able to decrease BP after systemic administration. In a previous study[14], we already showed with compound LNP 509 (injected directly in the brain) that an exclusive action on I_1Rs was sufficient to reduce BP. With the methylated analogue **4**, this information is confirmed but with a different mode of administration. Moreover, it is the first hypotensive IRC devoid of vasoconstrictive activity when administered intravenously. This compound therefore constitutes a promising prototype for the design of a novel generation of centrally acting antihypertensive drugs devoid of α_2-adrenergic-induced side

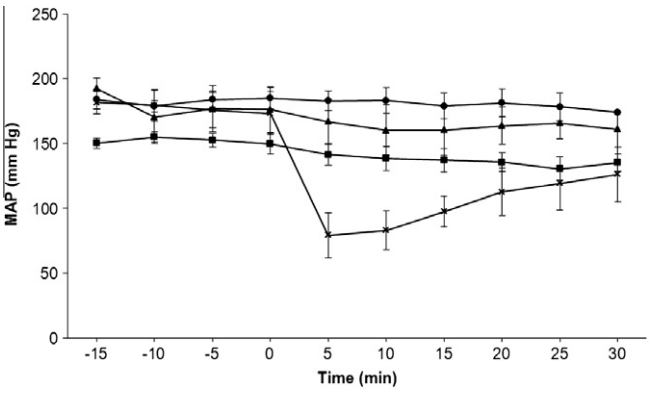


Figure 2. Effects on MAP of **4** (X), **7** (▲) and **10** (■) at doses of 1 mg/kg after intravenous administration (time = 0) to anaesthetized spontaneously hypertensive rats. Data are mean ± sem of five experiments. The control group (●) received an injection of saline in the usual volume (300 μL).

effects. So far, compound **4** has not been tested for sedation. The absence of initial vasoconstrictive effect (hypertensive phase) after its intravenous injection is a good predictor of the absence of α_2AR agonist activity. As such, it predicts the absence of any sedative effect at hypotensive doses. However, in case of development of compound **4** as an antihypertensive drug, this would have to be specifically checked.

At the same dose of 1 mg/kg compounds **7** and **10** did not significantly change BP (167 ± 41 mm Hg vs 185 ± 29 mm Hg for **7** and 141 ± 20 mmH g vs 149 ± 7 mm Hg for **10**). These compounds were also ineffective at a 10-fold higher dose (data not shown). The weaker I_1R affinity of compounds **7** and **10** could explain their inability to reduce BP. However alternative explanations such as inability in crossing the blood–brain barrier or more marked changes in the pharmacological profiles (e.g., from agonist to antagonist) could also account for their lack of hypotensive activity.

4. Conclusion

In this work, effects of IRC heterocycle methylation were studied. We have shown that this structural modification led to new derivatives selective for I_1Rs over α_2ARs and I_2BS. This result further confirms the different modes of binding to I_1Rs and α_2ARs of this family of ligand.

Moreover, an iodinated compound with high affinity and selectivity for I_1Rs has been obtained. The latter (**11**) is very useful in its radiolabelled and photolabelling forms to further study I_1Rs.[25,35]

Finally, we also synthesized the methylated analogue of clonidine (**4**) that still shows hypotensive activity despite its lack of α_2AR affinity. This agent is the first hypotensive imidazoline derivative described to be active after intravenous administration without any vasoconstriction. Compound **4** represents an interesting prototype for the development of novel centrally acting drugs with antihypertensive properties.

5. Experimental section

5.1. General information

All solvents were purified by standard methods before use. Flash chromatographies were performed on Merck silica gel Si 60 (40–63 μm) and TLC on Silica Gel 60 F_{254} (Merck). All organic layers were washed with brine and then dried with Na_2SO_4. Melting points were determined in open capillaries on a Gallenkamp apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were obtained with a Bruker AC 200 spectrometer. Solvents used were $CDCl_3$ ($CHCl_3$ at 7.26 ppm as reference) and D_2O (*t*-BuOH at 1.28 ppm as reference) for ^{1}H NMR and D_2O (*t*-BuOH at 70.4 and 30.3 ppm as reference) for ^{13}C NMR. The signals are described as: s (singlet); d (doublet); t (triplet); aro (aromatic) and br (broad). Chemical shifts (δ) are given in ppm and coupling constants (*J*) in Hz. The mass spectra were recorded using a LKB 2091 apparatus. The related intensities of the mass spectrum peaks are listed in parentheses. Elemental analyses were performed at the Service de microanalyse, Université de Strasbourg and at the Institut Charles Sadron, CNRS, Strasbourg. The HPLC system was a Waters platform with a 2767 sample manager, a 2525 pump, a photodiode array detector (190–400 nM). The column used was an Xterra C_{18} 3.5 μM (4.6 × 50 mm). The mobile phase consisted in an appropriate gradient of A and B. A was water with 0.05% of TFA and B was acetonitrile with 0.05% of TFA. Flow rate was 1 mL per min. All LCMS were performed at room temperature. The purities of final compounds **4**, **7**, **10** and **11**, were determined by elemental analysis and by HPLC. Compound **7** was not detected by UV-HPLC.

5.2. Synthetic procedures

5.2.1. *N*-(2,6-Dichlorophenyl)-formamide (2)

HCOOH (8 mL) and Ac_2O (20 mL) were heated at 40 °C for 2 h and 2,6-dichloroaniline **1** (15 g, 92.65 mmol) was added dropwise to this mixture. The latter was then heated at 40 °C for 4 h and stirred overnight at room temperature. The precipitate was filtered, rinsed with toluene, dried and crystallized from EtOH–benzene to afford **2** (12.84 g, 73%) as a white solid: mp 178 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ = 8.41 (br s, 1H, CHO), 7.40 (aro, 2H), 7.20 (aro + NH, 2H); MS *m*/*z* (%) 191(8), 189(13), 163(51), 161(82), 156(32), 154(100), 126(17), 125(20), 90(39).

5.2.2. 2,6-Dichloro-1-dichloroisocyano-benzene (3)

The formanilide derivative **2** (9.5 g, 50 mmol) was added slowly to a mixture of $SOCl_2$ (30 mL) and SO_2Cl_2 (7 g, 51.9 mmol). This solution was stirred and heated at 60 °C for 10 h under a nitrogen atmosphere. $SOCl_2$ was then removed by evaporation under reduced pressure and the residual mixture was distilled to afford **3** (7.28 g, 60%) as a clear yellow liquid: bp = 105 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.35 (aro, 2H), 7.09 (aro, 1H); MS *m*/*z* (%) 245(17), 243(35), 241(29), 210(31), 208(94), 206(100), 171(20), 147(20), 145(30), 109(32), 75(24).

5.2.3. (2,6-Dichloro-phenyl)-(4-methyl-4,5-dihydro-1*H*-imidazol-2-yl)-amine (4·HCl)

The isocyano derivative **3** (1 g, 4.12 mmol) was added to a solution of 1,2-diaminopropane (2.45 g, 33 mmol) in Et_2O (12 mL) and stirred for 12 h. This mixture was then diluted with 1 N HCl, washed with Et_2O, treated with 1 N NaOH until the pH ~13 and extracted with Et_2O (2 × 100 mL). This organic layer was washed, dried and evaporated to give an oil that was taken up in *n*-BuOH and evaporated under reduced pressure (in order to remove excess 1,2-diaminopropane). EtOH was added to the residual oil and this solution was treated with ethanolic HCl to form the hydrochloride salt. After evaporation and crystallization from 2-PrOH–Et_2O, compound **4**·HCl (0.75 g, 75%) was obtained as a white solid: mp 272–274 °C (dec.); ^{1}H NMR (200 MHz, D_2O): δ = 7.42–7.63 (aro, 3H), 4.25 (m, 1H, CH_2), 3.93 (m, 1H, CH), 3.42 (m, 1H, CH_2), 1.33 (d, 3H, CH_3, *J* = 6.1); 13C NMR (200 MHz, D_2O): δ = 158.1 (C_2), 135.0 (C aro), 131.7 (CH aro), 130.2 (C aro), 129.8 (CH aro), 52.4 (CH), 50.4 (CH_2), 20.4 (CH_3); MS *m*/*z* (%) 247 (11), 245(64), 244(15), 243(100), 230(15), 228(22), 174(18), 172(28), 44(96), 36(28). Anal. Calcd for $C_{10}H_{12}Cl_3N_3$: C 42.81, H 4.31, N 14.98. Found: C 42.59, H 4.25, N 14.70.

5.2.4. Dicyclopropylmethyl-carbamic acid phenyl ester (6)

To a solution of C,C-dicyclopropyl-methylamine **5**.HCl (5.9 g, 40 mmol) in H_2O (40 mL) was added dropwise Et_3N (10.1 g, 100 mmol) keeping the internal temperature below 10 °C. Phenyl chloroformate (6.20 g, 39.63 mmol) was then added and this solution was stirred 1 h at room temperature. The precipitate was filtered, washed with H_2O until neutral and dried in a vacuum desiccator charged with KOH to obtain **6** (7.9 g, 92%) as a white solid that was used for the next step without further purification: mp 81–82 °C (lit.[27] mp 92–94 °C); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.14–7.41 (aro, 5H), 5.06 (br s, 1H, NH), 2.84 (m, 1H, CH), 0.78–1.00 (m, 2H, CH × 2), 0.31–0.60 (m, 8H, CH_2 × 2); MS *m*/*z* (%) 188(10), 96(10), 95(100), 94(67), 81(11), 67(31), 55(11), 41(21).

5.2.5. Dicyclopropylmethyl-(4-methyl-4,5-dihydro-oxazol-2-yl)-amine (7·H_3PO_4)

To a solution of 2-amino-1-propanol (2.4 g, 32 mmol) in H_2O (20 mL) was introduced the carbamate **6** (4.63 g, 20 mmol). The mixture was refluxed for 2 h. After cooling to room temperature, the aqueous solution was extracted with CH_2Cl_2 (2 × 50 mL) and

the organic layer was washed with brine, dried and evaporated to give 2.59 g of a white solid. The latter was added to cold $CHCl_3$ (30 mL) and $SOCl_2$ (1.3 mL) was added to this solution. The mixture was stirred 1 h at room temperature under a nitrogen atmosphere. $CHCl_3$ was then removed under reduced pressure and the residual oil was mixed with H_2O (20 mL) and heated at reflux for 30 min. After cooling to room temperature, the aqueous solution was extracted with Et_2O (3 × 20 mL) and the organic layer was washed, dried and evaporated. The residual oil, after treatment with ethanolic H_3PO_4 gave **7**·H_3PO_4 (440 mg, 8%) as a white solid: mp 124–126 °C (2-PrOH–EtOAc); ^{1}H NMR (200 MHz, D_2O): δ = 4.39 (br s, 2H, H-5), 4.05 (m, 1H, H-4), 2.72 (br s, 1H, CH), 1.37 (d, 3H, CH_3, J = 5.7), 1.20 (m, 2H, CH × 2), 0.39–0.65 (m, 8H, CH_2 × 4); ^{13}C NMR (200 MHz, D_2O): δ = 161.2 (C_2), 77.1 (CH_2–O), 62.0 (CH–NH), 52.5 (C_4), 19.3 (CH_3), 14.7 (CH), 3.1 (CH_2), 2.1 (CH_2); MS m/z (%) 194(26), 179(59), 165(20), 153(20), 114(28), 101(33), 95(100), 79(46), 67(66), 41(45). Anal. Calcd for $C_{11}H_{18}N_2O{\cdot}H_3PO_4{\cdot}½C_3H_8O$: C 46.58, H 7.81, N 8.69. Found: C 46.12, H 8.21, N 8.32.

5.2.6. (2-Chloro-phenyl)-(5-methyl-4,5-dihydro-3*H*-pyrrol-2-yl)-amine (10·HCl)

To a solution of 5-methyl-2-pyrrolidinone (1.71 g, 17.27 mmol) and 2-chloro-aniline **8** (2.2 g, 17.26 mmol) in 1,2-dichloroethane (35 mL) was added $POCl_3$ (2.65 g, 17.27 mmol). The mixture was stirred at 60 °C for 4 h under a nitrogen atmosphere. After cooling to room temperature, H_2O (30 mL) was added and the mixture was made basic by addition of saturated aqueous K_2CO_3. This solution was extracted with CH_2Cl_2 (3 × 50 mL) and the organic layer was washed, dried and evaporated. The residual oil was purified by column chromatography on silica gel using 5% Et_3N in EtOAc as eluant and then crystallized from cyclohexane to give compound **10** (2.28 g, 65%) as a white solid: mp 92–93 °C. The hydrochloride salt, obtained by treatment with ethanolic HCl, was crystallized from 2-PrOH–Et_2O: mp 173–174 °C; ^{1}H NMR (200 MHz, D_2O): δ = 7.67 (aro, 1H), 7.50–7.53 (aro, 3H), 4.21 (m, 1H, H-5), 3.18 (m, 2H, H-3), 2.51 (m, 1H, H-4), 1.94 (m, 1H, H-4), 1.30 (d, 3H, CH_3, J = 7.1); RMN ^{13}C (200 MHz, D_2O): δ = 168.0 (C_2), 132.5 (C aro), 131.4 (CH aro), 131.3 (CH aro), 131.0 (C aro), 129.3 (CH aro), 128.6 (CH aro), 57.9 (C_5), 30.9 (C_3), 28.8 (C_4), 20.5 (CH_3); MS m/z (%) 210 (33), 209(22), 208(100), 207(29), 195(28), 193(89), 173(62), 166(16), 164(16), 36(32). Anal. Calcd for $C_{11}H_{14}Cl_2N_2$: C 53.89, H 5.76, N 11.43. Found C 54.11, H 5.89, N 11.26.

5.2.7. (2-Chloro-4-iodo-phenyl)-(5-methyl-4,5-dihydro-3H-pyrrol-2-yl)-amine (11·HCl)

5-Methyl-2-pyrrolidinone and 2-chloro-4-iodo-aniline **9** were subjected to the same procedure as described for compound **10** to give derivative **11** (37%): mp 126–128 °C (cyclohexane). The hydrochloride salt, obtained by treatment with ethanolic HCl, was crystallized from 2-PrOH–Et_2O: mp 225–228 °C (dec.); ^{1}H NMR (200 MHz, D_2O): δ = 8.09 (aro, d, 1H, J = 2), 7.87 (aro, dd, 1H, J = 8.4 and J = 2), 7.25 (aro, d, 1H, J = 8.4), 4.21 (m, 1H, H-5), 3.16 (m, 2H, H-3), 2.50 (m, 1H, H-4), 1.94 (m, 1H, H-4), 1.30 (d, 3H, CH_3, J = 6.2); RMN ^{13}C (200 MHz, D_2O): δ = 169.1 (C_2), 139.7 (CH aro), 138.4 (CH aro), 132.5 (C aro), 131.8 (C aro), 129.6 (CH aro), 94.7 (C aro), 58.0 (C_5), 31.03 (C_3), 28.7 (C_4), 20.4 (CH_3); MS m/z (%) 336(34), 334(100), 319(46), 321(15), 299(51). Anal. Calcd for $C_{11}H_{13}Cl_2IN_2$: C 35.61, H 3.53, N 7.55. Found: C 35.85, H 3.30, N 7.40.

5.3. Radioligand binding assays

5.3.1. I_1 Receptor binding assays

PC 12 cell membranes, prepared as described by Greney et al.[7], were used. These membranes (100 μg protein/250 μL) were incubated for 30 min at 25 °C with 0.5 nM [^{125}I]paraiodoclonidine (K_D of the radioligand) and increasing concentrations of drugs (10^{-12} to 10^{-3} M, 13 concentrations tested) in binding buffer (5 mM Tris Hepes, 0.5 mM EGTA, 0.5 mM EDTA, 0.5 mM $MgCl_2$, pH 7.7). Nonspecific binding was defined with 100 μM benazoline and represented 45% of the total binding.

5.3.2. I_2BS binding assays

The tissue was prepared as follows: renal cortex from male New Zealand rabbits was homogenized in ice-cold preparation buffer (50 mM Tris–HCl, 250 mM sucrose, pH 7.4). The homogenate was centrifuged at 500 g for 10 min at 4 °C. The supernatant was centrifuged at 28,000 g for 30 min and the resulting pellet was washed twice in binding buffer (50 mM Tris–HCl, pH 7.4). The membrane preparation was stored at −80 °C until used. These membranes (100 μg/250 μL) were incubated for 60 min at 25 °C with 5 nM [^{3}H]idazoxan and increasing concentrations of drugs tested in binding buffer (50 mM Tris–HCl, pH 7.4). Nonspecific binding was determined with 10 μM cirazoline.[37]

5.3.3. α_2-Adrenoceptor binding assays

CHO cells membranes were prepared as described by Newman-Tancredi et al.[38] These membranes (30 μg protein/mL for CHO-hα2A and CHO-hα2B, 100 μg protein/mL for CHO-hα2C) were incubated for 60 min at room temperature in binding buffer (33 mM Tris–HCl, 1 mM EDTA, pH 7.5) in a final volume of 500 μL containing 0.8, 1 or 2 nM [^{3}H]RX821002, respectively for hα2A-, hα2B- and hα2C-adrenoceptors. Nonspecific binding was defined with 10 μM phentolamine.

In these different assays, incubations were stopped by rapid filtration under vacuum through GF/B glass fiber filters followed by three successive washes with ice-cold binding buffer (for the I_1R binding assay, filters were incubated for 3 h in 0.03% PEI prior the filtration step). Radioactivity remaining on the filters was counted in a Packard Meriden Minaxi gamma counter (for I_1R binding assays) and in a Packard Tricarb counter (for I_2BS and α_2-AR binding assays). Results are given as the mean ± standard error mean (sem) of all experiments (at least three), each performed in triplicate. Competition experiments were analysed using the iterative nonlinear least-squares curve fitting program GraphPad. K_i were determined using the method of Cheng and Prussof.[39]

5.4. Cardiovasular experiments

5.4.1. General procedure

Male spontaneously hypertensive rats (Janvier, Le Genest St. Isle, France) weighing 250–350 g were anesthetized by an intraperitoneal injection of sodium pentobarbital (60 mg/kg); an additional injection of 20 mg/kg was performed immediately before the control period. The right jugular vein was catheterized to permit intravenous injections and the mean arterial blood pressure (MAP) was measured through a femoral arterial catheter connected to a Statham P23 Db transducer, which was connected to a pressure processor and recorder (BS272, Gould Electronique, France). The animals were tracheotomized, ventilated with room air (Rodent Ventilator, Harvard Apparatus, France) and immobilized with pancuronium bromide (1 mg/kg).

5.4.2. Drug injection

After a baseline period of 20 min, drugs (**4**, **7**, **10**) dissolved in saline or vehicle alone were slowly injected over a period of 20 s. Following administration, the cardiovascular parameters were continuous recorded during 30 min. Maximal effects were taken into consideration.

5.4.3. Accreditation

All the methods employed in this work are in accordance with the French law concerning experimentations on vertebrate laboratory animals (Décret 2001-464 from May 29, 2001 as a revision of the Décret 87-848, 1987) and according to European guidelines. L.M., J.F. and P.B. hold personal agreements from the Direction des Services Vétérinaires du Bas-Rhin, Agriculture Ministery, France (authorisation numbers 67-210 to LM, 67-2010 to JF, 67-249 to PB) which cover the protocols followed in the present study.

Data are given as mean ± s.e. mean. Homogeneity of initial cardiovascular parameters between groups was checked with an ANOVA.

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Supplementary data

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