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3-[(Imidazolidin-2-yl)imino]indazole ligands with selectivity for the α_2-adrenoceptor compared to the imidazoline I_1 receptor

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ABSTRACT

A series of 3-[(4,5-dihydroimidazolidin-2-yl)imino]indazoles has been synthesized as positional analogues of marsanidine, a highly selective α_2-adrenoceptor ligand. Parent compound **4a** and its 4-chloro (**4c**) and 4-methyl (**4d**) derivatives display α_2-adrenoceptor affinity at nanomolar concentrations (K_i = 39.4, 15.9 and 22.6 nM, respectively) and relatively high α_2/I_1 selectivity ratios of 82, 115 and 690, respectively. Evidence was obtained that these compounds act as partial agonists at α_{2A}-adrenoceptors. Compound **4d** with intrinsic activity comparable with that of marsanidine, but lower than that of clonidine, elicited pronounced cardiovascular effects in anesthetized rats at doses as low as 0.01 mg/kg iv
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1. Introduction

Imidazole and imidazoline-containing agents acting at α_2-adrenoceptors exhibit several important effects in vivo and could find potential applications as hypotensive, analgesic, sedative, anxiolytic, hemodynamic-stabilizing and organ-protective agents.[1–7] Other effects of these agents, mediated by central and peripheral autoreceptors located on noradrenergic nerve endings and by postsynaptic receptors on target cells, include stimulation of growth hormone secretion and decreased output of endocrine and exocrine secretory glands, such as decreased insulin secretion and decreased salivation.[8,9]

It has been hypothesised that compounds that selectively activate α_2-adrenoceptors, and not imidazoline I_1 receptors, would be likely to have decreased hypotensive and bradycardic effects compared with some non-selective agents that are now in clinical use,[10,11] such as clonidine and dexmedetomidine that may be considered as mixed α_2/I_1 agonists.

However, the above hypothesis appears to be uncertain, especially in view of the recent evidence[12,13] derived from experiments using D79N α_2-adrenoceptor transgenic mice (functional α_2-adrenoceptor 'knock-out' mice) that demonstrated that hypotensive compounds such as dexmedetomidine, brimonidine, clonidine, rilmenidine and moxonidine act as α_{2A}-adrenoceptor agonists in vivo to lower blood pressure. No evidence of I_1-imidazoline receptor-mediated hypotensive effects of these compounds was obtained. These observations did not support the imidazoline mode of action of these agents.[14]

Our previous investigations on α_2-adrenoceptor ligands led to the discovery of some highly selective 1-[(4,5-dihydroimidazolidin-2-yl)imino]indazoles[15] (Fig. 1, structure **A**, *marsanidine*). Based on analysis of the relative binding affinities of these compounds, we reasoned that one of the structural features which may contribute to α_2-adrenoceptor activity is the presence of an =*C*–*H* group in the indazole moiety which might participate in hydrogen bonding or stacking interactions with an aromatic ring side chain of the receptor protein (Fig. 1). Such a hypothesis was strongly supported by the observation that introduction of a methyl group at position 3 of the indazole ring was detrimental for α_2-adrenoceptor affinity (Fig. 1, structure **B**).

It was not obvious, however, what effect translocation of the iminoimidazolidine moiety might have with regard to α_2-adrenoceptor

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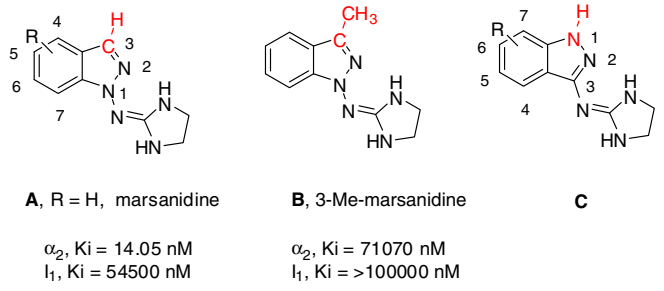


Figure 1. Structure of the selective α_2-adrenoceptor ligand **A** (marsanidine), its inactive and analogue **B** and positional isomer **C** showing common structural features: a C–H group and weakly acidic N–H group.

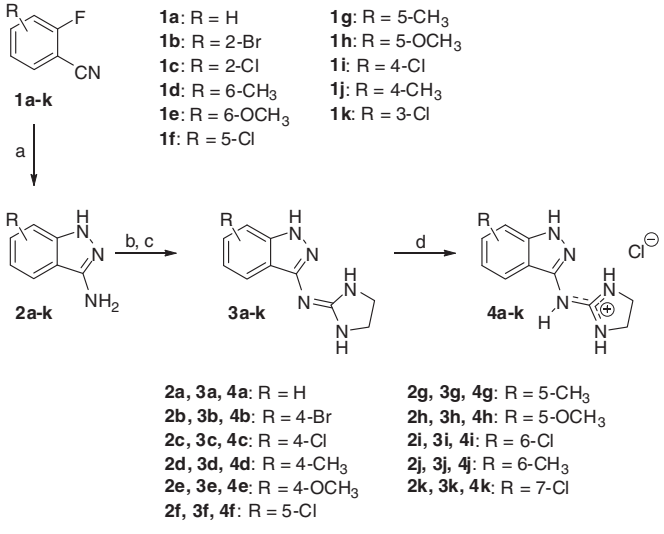
affinity, and therefore, we were interested in analogues of type **C** (Fig. 1) wherein the iminoimidazolidine moiety had been shifted to the 3-position of the indazole ring in order to obtain a new series of marsanidine analogues incorporating =*N*–*H* functionality at position 1. In the present work, the importance of the substitution pattern of the indazole ring for α_2-adrenoceptor affinity and selectivity has been explored. The pharmacological effects of such ligand modifications incorporated into the newly prepared positional analogues of type **C**, including functional characterization at recombinant α_{2A}-adrenoceptors and hemodynamic assessment in anesthetized rats, were also investigated.

2. Results and discussion

2.1. Chemistry

The known 3-aminoindazoles **2a**–**c**[16] and **2f**–**i**,[17] as well as the novel derivatives **2d–e** and **2j**–**k** were prepared according to previously described procedures by reacting the corresponding 2-fluorobenzonitriles with hydrazine[18] As shown in Scheme 1, 3-aminoindazoles **2a**–**k** reacted smoothly with *N*-*tert*-buthoxycarbonyl-4,5-dihydro-1*H*-imidazole[19] to give, after deprotection, the desired free bases **3a**–**k** in good yields. For pharmacological tests, water-soluble hydrochloride salts **4a**–**k** were prepared upon treatment of the free bases with methanolic HCl solution.

Structures of the free bases **3a**–**k** and the hydrochlorides **4a**–**k** were confirmed by elemental analysis, IR and NMR spectroscopy, as well as X-ray structure analysis of the compound **3a** (Fig. 2).



Scheme 1. Reagents and conditions: (a) $NH_2NH_2\cdot\cdot H_2O$, *n*-BuOH, 5 h, reflux; 42–79%; (b) *N*-Boc-2-methylthio-4,5-dihydro-1*H*-imidazole, acetic acid, 60–62 °C (oil bath), 16 h; (c) 15% Na_2CO_3/H_2O, crystallization from suitable solvent, 22–59%; (d) HCl/MeOH, 20–22 °C, 30 min, crystallization from suitable solvent, 41–53%.

Compound **3a** crystallizes as an imidazolidin-2-imine tautomer. The imidazolidine ring is in a flattened envelope conformation with the C13 atom forming a flap; the highest absolute value of the endocyclic torsion angle in the five-member ring is 17.5°. The indazole fragment of the molecule is virtually planar with the amino N1 atom slightly pyramidized. The indazole and imidazolidin-2-imine fragments are mutually twisted around the formally single C3–N10 bond by −18.7° and the conformation of **3a** is stabilized by an intramolecular N15–H···N2 hydrogen bond. The conformation of **3a** in solid state differs strongly from that of 4-chloromarsanidine,[15] where indazole and imidazolidin-2-imine fragments where nearly perpendicular and no intramolecular hydrogen bond was formed. In the crystals of **3a**, intermolecular N–H···N hydrogen bonds join the individual molecules into a two-dimensional polymeric structure and the amino N1–H group of the indazole ring plays an essential role in the recognition process. It acts as a hydrogen-bond donor to the imine N10 atom of the neighbouring molecule and also as a weak acceptor of the hydrogen bond from the N12–H group from the imidazolidine unit. In 4-chloromarsanidine the =C–H group, replacing the N1–H group of **3a**, is involved in an intermolecular C–H···π interaction to the benzene ring.

The molecular properties of marsanidine **A** (the most selective α_2/I_1 agent) and its positional analogue **3a** were also investigated by comparison of their 3D electrostatic potential maps.[20] As shown in Figure 3, electrostatic potential maps of both free base **A** and free base **3a** show superimposable negative wells positioned around the aromatic indazole ring and nitrogen atoms of the 2-iminoimidazolidine moiety. Therefore, a similar mode of interactions with α_2-adrenoceptors may be assumed by marsanidine-like ligands and their 3-(2-iminoimidazolidine) analogues **3**. However, in the case of **3a** the negative potential is more pronounced, especially in close proximity to the indazole N2 nitrogen atom and the exocyclic nitrogen atom of the 2-iminoimidazolidine moiety. This observation is further confirmed by the more basic character of **3a**[21] compared to marsanidine (pK_a = 8.02 vs 6.32). Consequently, a difference of 1.7 log units when comparing the basicity of **3a** to marsanidine should influence the pharmacokinetic profiles of the newly prepared compounds. At physiological pH, marsanidine with a pK_a of 6.32 exists preferably in neutral free base form (92%) which, in turn, facilitates its ability to cross the blood–brain barrier (BBB). Instead, the compound **3a** with pK_a = 8.02 bears resemblance to the well known aryliminoimidazolidines such as clonidine, and is mostly ionized at physiological pH (only 19% in non-ionized form at pH 7.4).

2.2. Radioligand binding assays

Radioligand binding experiments were conducted using crude P_2 rat brain membranes for both α_2-adrenoceptors and imidazoline I_2 receptors and crude P_2 rat kidney membranes for α_1-adrenocep-

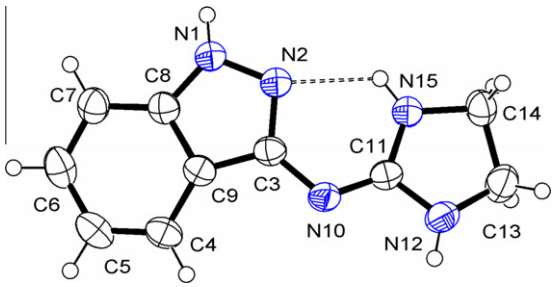


Figure 2. Molecular structure of **3a** with atom labelling scheme and displacement ellipsoids shown at the 50% probability level. The intramolecular N–H···N hydrogen bond is shown with a dashed line.

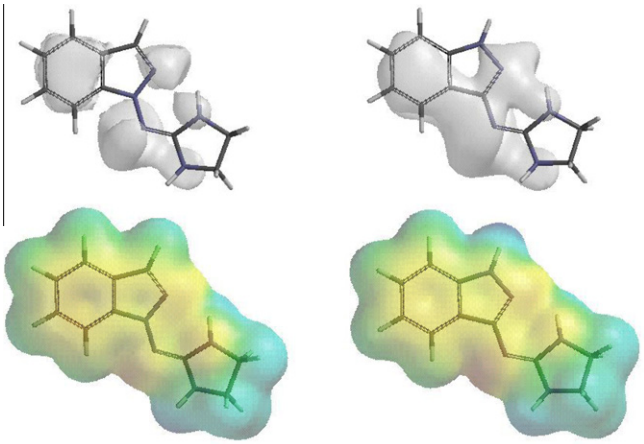


Figure 3. Comparison of the electrostatic potential maps of marsanidine **A** (*left*) and its positional analogue **3a** (*right*). Electrostatic potential map isocontoured at −10 kcal/mol (*top*) and electrostatic potential mapped onto the surface of electron density (*bottom*).

tors and I_1 receptors. Equilibrium dissociation constants (K_i) were determined by the method of Cheng and Prusoff[22] and the resulting values are given as the mean ± sem for three or four separate experiments.

As shown in Table 1, the unsubstituted compound **4a** showed good affinity for α_2-adrenoceptors with K_i = 39.4 nM and a moderate I_1/α_2 selectivity ratio of 82. The 4-Cl and 4-CH_3-substituted indazoles **4c** and **4d** displayed enhanced α_2-adrenoceptor affinities (K_i = 15.9 and 20.0 nM, respectively) and higher selectivity with respect to I_1 receptors (I_1/α_2 selectivity ratios of 115 and 690, respectively). Worth noting is a noticeable difference in binding between **4b** (4-Br) and **4c** (4-Cl) at the α_2-receptor (6420 vs 15.9 nM). This result, however, is difficult to rationalize based on both the electronic properties (similar electronegativity and identical Hammet constant σ), and steric factors, since the compound **4e** bearing 4-OCH_3 group is much more active (K_i = 196 nM). Perhaps, the hydrophobic pocket in this region appears to be size-limited and bromine atom is least favourable at the C4 position.

On the other hand, 5-substituted analogues **4f–h** showed affinities about 6–285 times lower at both α_2 and I_1 receptors, while substitution at the indazole C-6 and C-7 positions (entries **4i–k**) proved to be less detrimental, especially at α_2-adrenoceptors. It should be pointed out that the most active compounds at α_2-adrenoceptors, **4c** and **4d**, also displayed good affinities to α_1-adrenoceptors (K_i = 69.3 and 46.9 nM, respectively), while the 7-Cl congener **4k** exhibited relatively high affinity for the imidazoline I_2 receptor (K_i = 30.8 nM).

In Table 1 the selected results obtained for analogous 1-[(imidazolidin-2-yl)imino]indazoles of type **A** are included (values given in parentheses). From comparison of the relevant data it can be concluded that translocation of the 2-iminoimidazolidine moiety from position 1 to position 3 of the indazole ring yields compounds with high or moderate binding affinity to α_2-adrenoceptors and relatively high I_1/α_2 selectivity ratios, depending on the substitution pattern at the indazole ring.

2.3. Functional [^{35}S]GTPγS binding assays

Ligands **4a**, **4c**, **4d** and **4k** that displayed affinities towards rat brain α_2-adrenoceptors within the nanomolar range were subjected to [^{35}S]GTPγS binding experiments to determine their intrinsic activity and agonist potency.[23–25] For SAR purposes, marsanidine and its 4-Cl, 7-Me and 7-Cl derivatives representing an indazole series of type **A** were also included.

All of the investigated compounds were found to stimulate binding of [^{35}S]GTPγS to CHO cell membranes expressing recombinant human α_{2A}-adrenoceptors and showed typical agonist dose–response plots. Table 2 presents their agonist potencies expressed as EC_{50} values and their relative efficacy compared to the natural full agonist noradrenaline.

When compared to the full agonist noradrenaline, the reference drugs dexmedetomidine and clonidine as well as the two series of indazole derivatives were identified as partial agonists. Agonist potencies of both series were in the submicromolar range with the newly prepared **4d** being the most potent agonist (EC_{50} = 14 nM). On the other hand, in both series, the highest intrinsic activity was displayed by congeners containing a Cl-substituent at a similar position relative to the iminoimidazolidine moiety, that is, by 4-Cl-marsanidine and 7-chloro-3-[(4,5-dihydroimidazol-2-yl) imino] indazole **4k**. Moreover, their intrinsic activities were found to be on the same level as those of clonidine and dexmedetomidine (efficacy estimates relative to noradrenaline were 62%, 66%, 53% and 60%, respectively).

Table 1
Binding affinities of 3-[(imidazolidin-2-yl)imino]indazole hydrochlorides **4a–k** at α-adrenorceptors and imidazoline receptors

Compound		Binding affinities[a]				α_2-selectivity[d]
No.	R	α_1 K_i (nM)[b]	α_2 K_i (nM)[b]	I_1 IC_{50} (nM)[c]	I_2 K_i (nM)[b]	
4a (13a)	H (H)	NT[e] (NT)	39.4 ± 7.7 (14)	3219.0 ± 271 (54,500)	133.6 ± 19.6 (16,900)	82 (3879)
4b	4-Br	984 ± 142	6420 ± 829	7560 ± 2550	5380 ± 916	1
4c	4-Cl (7-Cl)[f]	69.3 ± 19.8 (1010)	15.9 ± 2.3 (30.3)	1840.0 ± 580 (46,800)	45.2 ± 14.2 (15,000)	115 (1544)
4d (13k)	4-CH_3 (7-CH_3)	46.5 ± 1.6 (NT)	22.6 ± 6.5 (53.5)	32,500 ± 2100 (387)	132.7 ± 8.3 (2520)	690 (7.2)
4e	4-OCH_3	196 ± 11.8	748 ± 33.7	25,600	167.0 ± 18.9	34
4f	5-Cl	121.0 ± 8.8	10600.0 ± 3700	>100,000	93000.0	>9.4
4g (13i)	5-CH_3 (6-CH_3)	29,000 (NT)	11400.0 (49)	21,200 (NT)	10,200 (2520)	2
4h (13j)	5-OCH_3 (6-OCH_3)	244 ± 58.0 (NT)	1460 ± 310 ((96)	>100,000 (NT)	9910.0 ± 360 (33,640)	>68
4i (13h)	6-Cl (5-Cl)	386 ± 27.0 (NT)	502 ± 110 (114)	2010 ± 430 (16,980)	410 ± 73.0 (20,620)	4 148
4j (13g)	6-CH_3 (5-CH_3)	508 ± 166 (NT)	176 ± 37.4 (20,310)	16,600 ± 4770 (7159)	325 ± 198	94.3 (459)
4k (13e)	7-Cl (4-Cl)	684 ± 48.3 (NT)	133 ± 15.3 (61)	4010 ± 599 (31,460)	30.8 ± 2.9 (11,080)	30 (510)

[a] Values shown are mean ± sem from 3 to 4 experiments, otherwise values represent the mean of two separate assays. In parentheses the corresponding values for 1-substituted indazoles of type **A** are shown (see Ref. 15).

[b] K_i affinity values for α_1-adrenoreceptors, α_2-adrenoreceptors and I_2 imidazoline binding sites were assessed by measuring the ability of the test compounds to displace [^{3}H]prazosin (rat brain membranes), [^{3}H]RX821002 (rat brain membranes) or [^{3}H]2BFI (rat brain membranes), respectively.

[c] Molar concentration of the test compounds that displaces 50% of specifically bound [^{3}H]clonidine (rat kidney membranes).

[d] α_2-Selectivity was determined from the ratio of the equilibrium constants: I_1 IC_{50}/α_2 K_i.

[e] NT: not tested.

[f] The previously not described analogue 7-chloro-1-[(imidazolidin-2-yl)imino]indazole hydrochloride was prepared and tested according to the procedures presented in Ref. 15; mp: 223–224 °C; ^{1}H NMR (DMSO-d_6, 200 MHz): δ = 12.55 (br s, 1H), 9.09 (s, 2H), 8.37 (s, 1H), 7.86 (d, J = 7.9 Hz, 1H), 7.61 (d, J = 7.3 Hz, 1H), 7.29 (t, 1H), 3.75 (s, 4H).

Table 2
Characterization of [^{35}S]GTPγS binding to CHO cell membranes expressing recombinant human α_{2A}-adrenoceptors: estimates of agonist potency (EC_{50}) and intrinsic activity relative to the natural full agonist noradrenaline

Ligand	EC_{50} (nM)	Intrinsic activity (% of noradrenaline)
Noradrenaline	110 ± 14	100
Dexmedetomidine	1.5 ± 0.19	60 ± 2
Clonidine	28 ± 4.3	53 ± 3
Marsanidine	160 ± 66	18 ± 2
4-Cl-Marsanidine	180 ± 38	62 ± 6
7-Me-Marsanidine	37 ± 9.0	32 ± 5
7-Cl-Marsanidine[a]	15 ± 1.9	48 ± 3
4a	82 ± 16	55 ± 6
4c	240 ± 140	13 ± 1
4d	14 ± 2.1	40 ± 5
4k	73 ± 10	66 ± 3

Values shown are mean ± sem from 3 to 16 independent experiments.
[a] 7Cl-Marsanidine has been prepared according to general procedure described in Ref. 15.

2.4. In vivo cardiovascular effects

Compounds **4c**, **4d** and **4k**, with relatively high affinity for α_2-adrenoceptors (Table 1) and clonidine-like partial agonist activity at α_{2A}-adrenoceptors (Table 2) were evaluated in anaesthetized rats for possible cardiovascular effects.[26] As shown in Table 3, neither **4c** nor **4k** exerted significant effects on mean arterial blood pressure after intravenous infusion at doses up to 0.1 mg/kg. Much higher cardiovascular activity was observed for the 4-methylated congener **4d** (Figs. 4 and 5). The maximal hypotensive and negative chronotropic effect of 0.1 mg/kg **4d** administration reached ΔMAP = −53.5 mmHg and ΔHR = −132 bpm, respectively(Tables 3 and 4). Similar structure–activity relationships were previously found for the 1-substituted indazole series **A**, wherein the greatest hypotensive activity was noted for the congener bearing a methyl group at the *peri* position, that is, 7-Me-marsanidine.[15]

Weaker in vivo hemodynamic effects of the systemically administered compounds **4c** and **4k** compared to **4d** (Fig. 4) could not be explained by their inability to cross the BBB, as the calculated lipophilicity of the former compounds is comparable to that of **4d** (log *P* (Ghose–Crippen) = 2.07 vs 2.0). Since **4d** exhibited the highest I_1/α_2 selectivity ratio of the tested compounds, 550 (Table 1), and proved to be a potent agonist of the α_{2A}-adrenoceptor subtype (EC_{50} = 14 nM, Table 2), we may conclude that the hypotensive effect of this compound is best explained by activation of α_{2A}-adrenoceptors.

As to the blood pressure effects elicited by the active compounds **4d** and **4k** (Fig. 4), a classical biphasic response characteristic for activation of α_2-adrenoceptors was observed for compound **4k**, while in the case of the more potent **4d**, a hypertensive phase was much less pronounced. The initial hypertensive phase is probably at least in part mediated by vascular α_{2B}-adrenoceptors (but α_1-adrenoceptors may also contribute), whereas the long-lasting hypotension is mediated by central α_{2A}-adrenoceptors.[9] Thus, compound **4d** may in functional terms prefer the α_{2A}-adrenoceptor subtype as compared to **4k** and the previously studied marsanidine analogues.[15] Further receptor binding and functional studies on the cloned α_2-adrenoceptor subtypes will be necessary to examine this preference in detail.

3. Conclusion

We have here described a facile synthesis of 3-[(4,5-dihydroimidazolidin-2-yl)imino]indazoles, which constitute a new series of positional analogues of the previously reported highly α_2-adrenoceptor selective 1-[(4,5-dihydroimidazolidin-2-yl)imino]indazoles (marsanidine-like ligands). Compounds **4a** and **4c**–**d** showed nanomolar affinities to α_2-adrenoceptors in in vitro receptor binding experiments and relatively high I_1/α_2 selectivity ratios ranging from 82 to 690. In terms of pharmacological activity, compounds **4a**, **4c**, **4d** and **4k** are partial α_2-adrenoceptor agonists as determined in functional [^{35}S]GTPγS binding experiments in vitro. When systemically administered to anaesthetized rats, the compound **4d** elicited pronounced hemodynamic effects and may prove useful in the treatment of hypertension. On the other hand, the weak partial agonist **4c** was shown to possess very weak hypotensive properties. Such a pharmacological profile may prove beneficial for therapeutic applications other than the treatment of hypertension.

4. Experimental

4.1. Chemistry

Melting points were measured on a Büchi 535 apparatus and are not corrected. IR spectra were taken in KBr pellets on a Perkin–Elmer FTIR 1600 spectrometer. NMR spectra were recorded on a Varian Gemini 200 or a Varian Unity 500 apparatus. ^{1}H and ^{13}C chemical shifts were measured relative to the residual solvent signal at 2.50 ppm and 39.5 ppm (DMSO-d_6). Mass spectra were recorded on a FinniganMAT 95 mass spectrometer at 70 eV. The results of elemental C, H, N analyses of all newly prepared compounds were within ±0.4% of the theoretical values. The following compounds were obtained according to previously described procedures: 3-aminoindazoles (**2a–c**),[16] (**2f–i**)[17] by reacting the commercially available 2-fluorobenzonitrile **1a–c**, **f–i** with 98% hydrazine hydrate.[18] The same procedure was applied to the synthesis of the previously not described 3-aminoindazole derivatives **2d**–**e** and **2j**–**k**. *N*-*tert*-Buthoxycarbonyl-4,5-dihydro-1*H*-imidazole were obtained by reacting 2-methylthio-2-imidazoline hydroiodide with di-*tert*-butyl dicarbonate.[19]

4.2. General procedure for the preparation of 3-aminoindazoles (2d–e, j–k)

The title compounds were obtained according to the procedure described by Vasudevan et al.[18] A solution of the commercially available 2-fluorobenzonitrile (**1d–e**, **j–k**) (22.0 mmol) and 98% hydrazine hydrate (90.0 mmol) in *n*-butanol (15 ml) was stirred

Table 3
The maximal hypotensive effect of synthesized compounds at 0.01, 0.1 and 1.0 mg/kg in anesthetized rats

Compound	0.01 mg/kg		*p*	0.1 mg/kg		*p*	1.0 mg/kg	
	Δ MAP (mmHg)	(*n*)		Δ MAP (mmHg)	(*n*)		Δ MAP (mmHg)	(*n*)
4c	−10.8 ± 4.2	3	NS	−10.8 ± 2.9	4	NS	−23.6 ± 7.0	4
4d	−17.1 ± 1.9	4	<0.001	−53.5 ± 2.6	4	<0.001	−22.0 ± 2.6	4
4k	−12.2 ± 4.5	3	NS	−21.7 ± 3.7	5	NS	−37.2 ± 6.7	5

Values are mean ± sem; *n*, number of animals; *p*, significance; NS, not statistically significant; ΔHR, the difference of heart rate (HR) between the moment of maximal hypotensive effect and the baseline (time 0 of experiment).

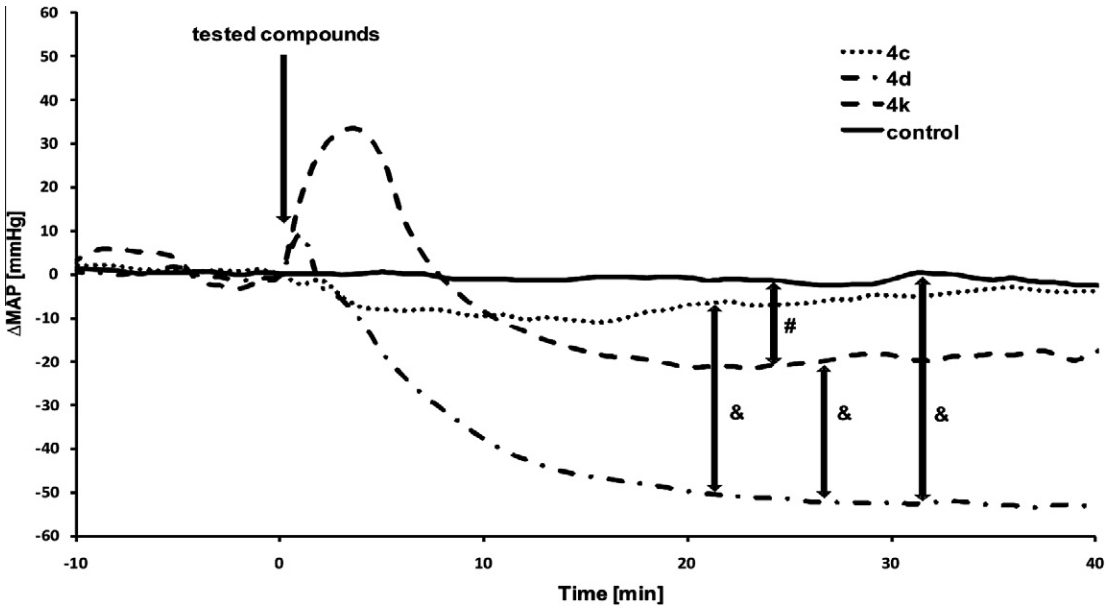


Figure 4. Effect of 0.1 mg/kg of **4c**, **4d** and **4k** on ΔMAP (calculated as the difference of MAP from baseline) in rats. Each point represents the mean value of ΔMAP for four or five experiments. Comparisons were made using ANOVA with repeated measures and Fisher test. Significances (&) p <0.001 were found for comparisons of **4d** versus **4c**, **4d** versus **4k** and **4d** versus **control** groups. Significance (#) p <0.01 was found for comparison of **4k** versus **control** group.

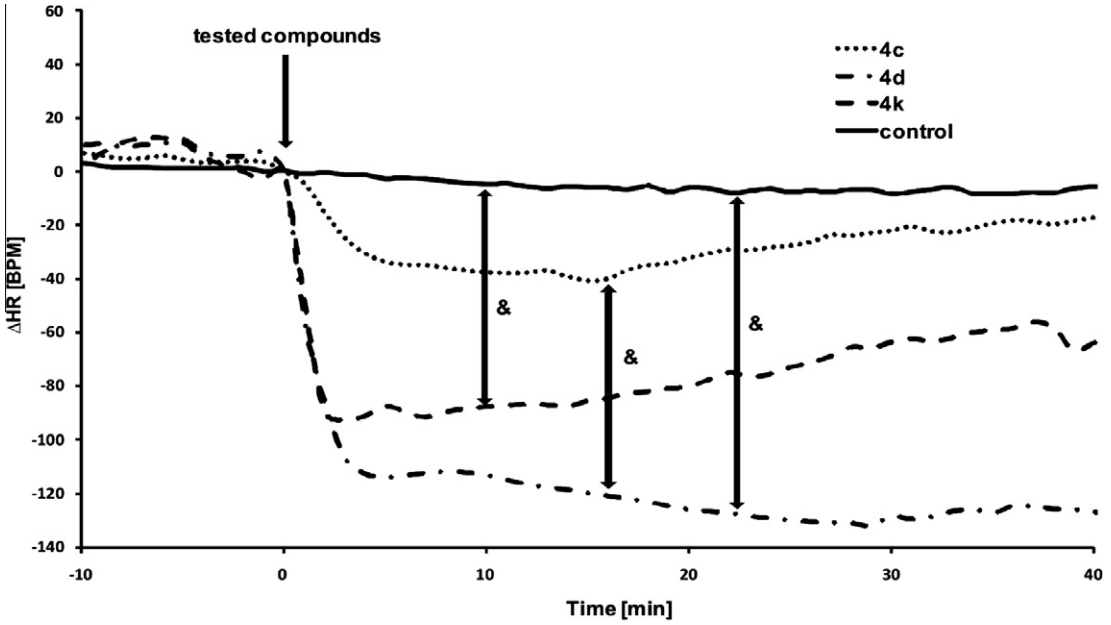


Figure 5. Effect of 0.1 mg/kg of **4c**, **4d** and **4k** on ΔHR (calculated as the difference of HR from baseline) in rats. Each point represents the mean value of ΔHR for four or five experiments. Comparisons were made using ANOVA with repeated measures and Fisher test. Significances (&) p <0.001 were found for comparisons of **4d** and **4k** versus **control** group and of **4k** versus **4c** group.

Table 4
The maximal negative chronotropic effect of synthesized compounds at 0.01, 0.1 and 1.0 mg/kg in anesthetized rats

Compound	0.01 mg/kg		p	0.1 mg/kg		p	1.0 mg/kg	
	Δ HR (bpm)	(n)		Δ HR (bpm)	(n)		Δ HR (bpm)	(n)
4c	−37 ± 20	3	NS	−40 ± 15	4	NS	−68 ± 25	4
4d	−43 ± 8	4	<0.001	−132 ± 10	4	NS	−155 ± 13	4
4k	−35 ± 11	3	<0.01	−92 ± 22	5	NS	−117 ± 23	5

Values are mean ± sem; n, number of animals; p, significance; NS, not statistically significant; ΔHR, the difference of heart rate (HR) between the moment of maximal hypotensive effect and the baseline (time 0 of experiment).

under reflux for 7 h. After cooling to room temperature, the resulting mixture was concentrated under reduced pressure to a volume of 5 ml, and then water was added. The solid product that was precipitated was filtered, washed with water, dried and purified by crystallization from methanol. In this manner, the following compounds were obtained.

4.2.1. 3-Amino-4-methylindazole (2d)

Yield: 2.1 g (65%). Beige solid; mp: 153–156 °C. IR (KBr): 3454, 3335, 3183 (NH_2, NH), 2964, 2922 (CH), 1609 (δ NH_2, C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 500 MHz): δ 2.59 (s, 3H, CH_3), 4.92 (s, 2H, NH_2), 6.60 (d, J = 6.8 Hz, 1H, aromat.), 7.04–7.07 (m, 2H, aromat.), 11.41 (s, 1H, NH). Anal. Calcd for $C_8H_9N_3$ (147.18): C, 65.29; H, 6.16; N 28.25. Found: C, 65.32; H, 5.98; N, 28.01.

4.2.2. 3-Amino-4-methoxyindazole (2e)

Yield: 1.6 g (44%). Beige solid; mp: 163–166 °C. IR (KBr): 3467, 3437, 3184 (NH_2, NH), 3006, 2955 (CH), 1625 (δ NH_2), 1600 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 200 MHz): δ 3.84 (s, 3H, OCH_3), 4.97 (s, 2H, NH_2), 6.30 (d, J = 7.7 Hz, 1H, aromat.), 6.76 (d, J = 8.1 Hz, 1H, aromat.), 7.09 (t, 1H, aromat.), 11.39 (s, 1H, NH). Anal. Calcd for $C_8H_9N_3O$ (163.18): C, 58.88; H, 5.56; N, 25.75. Found: C, 58.71; H, 5.23; N, 25.42.

4.2.3. 3-Amino-6-methylindazole (2j)

Yield: 2.5 g (79%). Beige solid; mp: 199–201 °C. IR (KBr): 3444, 3309, 3194 (NH_2, NH), 2916, 2858 (CH), 1625 (δ NH_2, C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 200 MHz): δ 2.35 (s, 3H, CH_3), 5.23 (s, 2H, NH_2), 6.71 (d, J = 8.1 Hz, 1H, aromat.), 6.99 (s, 1H, aromat.), 7.53 (d, J = 8.1 Hz, 1H, aromat.), 11.17 (s, 1H, NH). Anal. Calcd for $C_8H_9N_3$ (147.18): C, 65.29; H, 6.16; N, 28.55. Found: C, 65.01; H, 5.98; N, 28.32.

4.2.4. 3-Amino-7-chloroindazole (2k)

Yield: 1.92 g (52%). White solid; mp: 187–188 °C. IR (KBr): 3402, 3169, 3148 (NH_2, NH), 2941, 2854 (CH), 1626 (δ NH_2, C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 200 MHz): δ 5.51 (s, 2H, NH_2), 6.90 (t, 1H, aromat.), 7.30 (d, J = 7.3 Hz, 1H, aromat.), 7.67 (d, J = 8.0 Hz, 1H, aromat.), 11.84 (s, 1H, NH). Anal. Calcd for $C_7H_6ClN_3$ (167.60): C, 50.17; H, 3.61; N, 25.07. Found: C, 50.01; H, 3.36; N, 24.89.

4.3. General procedure for the preparation of 3-[(imidazolidin-2-ylo)imino]indazoles (3a–k)

A suspension of the appropriate 3-aminoindazole (**2a–k**) (3.2 mmol) and *N*-*tert*-butoxycarbonyl-2-methylthio-4,5-dihydro-1*H*-imidazole (0.83 g, 3.84 mmol) in acetic acid (2 ml) was stirred at 60–62 °C (oil bath) for 16 h and then the solvent was evaporated under reduced pressure. The viscous residue was treated with water (7 ml) and to the resulting solution or mixture was added dropwise 15% aqueous Na_2CO_3 solution to pH 9.5–10. The precipitate thus obtained was filtered, washed with water, dried and purified by crystallization from suitable solvent. In this manner, the following compounds were obtained.

4.3.1. 3-[(Imidazolidin-2-yl)imino]indazole (3a)

Yield: 0.35 g (54%). White solid; mp: 218–221 °C. IR (KBr): 3390, 3335 (NH), 2890, 2745 (CH), 1615 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 200 MHz): δ 3.46 (br s, 4H, CH_2), 6.65 (br s, 1H, NH), 6.93 (t, 1H, aromat.), 7.20–7.52 (m, 2H, aromat.), 7.41 (br s, 1H, NH), 7.59 (d, J = 7.8 Hz, 1H, aromat.), 11.75 (br s, 1H, NH). MS (EI) m/z = 201.1 (M^+, 100), 144.1 (17), 118.1 (12). Anal. Calcd for $C_{10}H_{11}N_5$ (201.23): C, 59.69; H, 5.51; N 34.80. Found: C, 59.32; H, 5.21; N, 34.92.

4.3.2. 4-Bromo-3-[(imidazolidin-2-yl)imino]indazole (3b)

Yield: 0.20 g (22%). White solid; mp: 200–203 °C (MeOH). IR (KBr): 3335, 3195, 3155 (NH), 2940, 2885 (CH), 1630, 1605 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 200 MHz) δ: 3.44 (s, 4H, CH_2), 6.89 (br s, 1H, NH), 7.07–7.14 (m, 2H, aromat.), 7.22 (br s, 1H, NH), 7.26–7.31 (m, 1H, aromat.), 12.09 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 50 MHz) δ: 41.8 (two overlapping signals), 109.1, 114.6, 115.7, 122.3, 127.1, 142.1, 151.8, 159.1. Anal. Calcd for $C_{10}H_{10}BrN_5$ (280.12): C, 42.88; H, 3.60; N, 25.00. Found: C, 42.62; H, 3.43; N, 25.32.

4.3.3. 4-Chloro-3-[(imidazolidin-2-yl)imino]indazole (3c)

ZYield: 0.32 g (42%). White solid; mp: 227–229 °C (EtOH). IR (KBr): 3400, 3350, 3120 (NH), 3085, 2880 (CH), 1610 (C=N) cm^{-1}. Widmo ^1H NMR (DMSO-d_6, 200 MHz) δ: 3.45 (s, 4H, CH_2), 6.87–6.91 (m, 1H, aromat.), 7.00 (br s, 2H, NH), 7.12–7.25 (m, 2H, aromat.), 12.09 (br s, 1H, NH). Anal. Calcd for $C_{10}H_{10}ClN_5$ (235.67): C, 50.96; H, 4.28; N, 29.72. Found: C, 50.63; H, 3.96; N, 29.58.

4.3.4. 3-[(Imidazolidin-2-yl)imino]-4-methylindazole (3d)

Yield: 0.41 g (59%). White solid; mp: 199–203 °C (acetonitrile). IR (KBr): 3390, 3345 (NH), 3090, 2945, 2880 (CH), 1610 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 500 MHz) δ: 2.71 (s, 3H, CH_3), 3.54 (br s, 4H, CH_2), 6.27 (br s, 1H, NH), 6.65–6.67 (m, 1H, aromat.), 7.06–7.10 (m, 2H, aromat.), 7.62 (br s, 1H, NH), 11.69 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 19.2, 41.9, 43.9, 107.4, 117.6, 119.7, 126.8, 133.8, 141.8, 153.8, 159.8. Anal. Calcd for $C_{11}H_{13}N_5$ (215.25): C, 61.38; H, 6.08; N, 32.53. Found: C, 61.02; H, 5.73; N, 32.21.

4.3.5. 3-[(Imidazolidin-2-yl)imino]-4-methoxyindazole (3e)

Yield: 0.23 g (31%). Beige solid; mp: 266–268 °C (DMF). IR (KBr): 3390, 3340, 3160 (NH), 2960, 2880 (CH), 1635, 1615 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 500 MHz) δ: 3.41 (br s, 4H, CH_2), 3.80 (s, 3H, OCH_3), 6.33 (d, J = 7.8 Hz, 1H, aromat.), 6.56 (br s, 1H, NH), 6.83 (d, J = 8.3 Hz, 1H, aromat.), 7.12 (t, 1H, aromat.), 7.59 (br s, 1H, NH), 11.78 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 41.9, 44.1, 55.6, 99.1, 102.8, 108.9, 127.9, 143.8, 152.5, 155.8, 160.1. Anal. Calcd for $C_{11}H_{13}N_5O$ (231.25): C, 57.13; H, 5.67; N, 30.28. Found: C, 56.87: H, 5.41; N, 29.95.

4.3.6. 5-Chloro-3-[(imidazolidin-2-yl)imino]indazole (3f)

Yield: 0.22 g (29%). White solid; mp: 272–274 °C (DMF/MeOH). IR (KBr): 3330, 3140 (NH), 2880 (CH), 1620 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 200 MHz) δ: 3.47 (br s, 4H, CH_2), 6.75 (br s, 1H, NH), 7.23 (d, J = 8.8 Hz, 1H, aromat.), 7.32 (d, J = 8.8 Hz, 1H, aromat.), 7.42 (br s, 1H, NH), 7.52 (s, 1H, aromat.), 11.95 (br s, 1H, NH). Anal. Calcd for $C_{10}H_{10}ClN_5$ (235.67): C, 50.96; H, 4.28; N, 29.72. Found: C, 50.62; H, 3.97; N, 29.67.

4.3.7. 3-[(Imidazolidin-2-yl)imino]-5-methylindazole (3g)

Yield: 0.27 g (39%). White solid; mp: 236–239 °C (EtOH). IR (KBr): 3395, 3300 (NH), 3080, 2880 (CH), 1615 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 200 MHz) δ: 2.35 (s, 3H, CH_3), 3.43 (br s, 4H, CH_2), 6.60 (br s, 1H, NH), 7.07 (d, J = 8.2 Hz, 1H, aromat.), 7.18 (d, J = 8.2 Hz, 1H, aromat.), 7.36 (s, 1H, aromat.), 7.57 (br s, 1H, NH), 11.60 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 50 MHz) δ: 21.2, 42.0 (two overlapping signals), 109.4, 119.1, 119.8, 126.8, 128.2, 139.8, 152.0, 159.7. Anal. Calcd for $C_{11}H_{13}N_5$ (215.25): C, 61.38; H, 6.08; N, 32.53. Found: C, 61.11; H, 5.86; N, 32.50.

4.3.8. 3-[(Imidazolidin-2-ylo)imino]-5-methoxyindazole (3h)

Yield: 0.16 g (22%). Beige solid; mp: 219–223 °C (*i*-PrOH). IR (KBr): 3395, 3360 (NH), 3050, 2945, 2880 (CH), 1625 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 500 MHz) δ: 3.57 (br s, 4H, CH_2), 3.75 (s, 3H, OCH_3), 6.51 (br s, 1H, NH), 6.93–6.97 (m, 2H, aromat.), 7.21 (d, J = 5.4 Hz, 1H, aromat.), 7.56 (br s, 1H, NH), 11.64 (br s, 1H, NH).

Anal. Calcd for $C_{11}H_{13}N_5O$ (231.25): C, 57.13; H, 5.67; N, 30.28. Found: C, 56.89; H, 5.32; N, 30.11.

4.3.9. 6-Chloro-3-[(imidazolidin-2-yl)imino]indazole (3i)

Yield: 0.32 g (42%). White solid; mp: 280–282 °C (DMF). IR (KBr): 3435, 3375 (NH), 2955, 2890 (CH), 1625, 1610 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 500 MHz) δ: 3.48 (br s, 4H, CH_2), 6.73 (br s, 1H, NH), 6.93 (d, J = 8.5 Hz, 1H, aromat.), 7.34 (s, 1H, aromat.), 7.50 (br s, 1H, NH), 7.57 (d, J = 8.5 Hz, 1H, aromat.), 11.91 (br s, 1H, NH). Anal. Calcd for $C_{10}H_{10}ClN_5$ (235.67): C, 50.96; H, 4.28; N, 29.72. Found: C, 50.67; H, 3.99; N, 29.89.

4.3.10. 3-[(Imidazolidin-2-yl)imino]-6-methylindazole (3j)

Yield: 0.31 g (45%). White solid; mp: 265–267 °C (DMF). IR (KBr): 3385, 3345 (NH), 3080, 2970 (CH), 1610 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 200 MHz) δ: 2.37 (s, 3H, CH_3), 3.45 (br s, 4H, CH_2), 6.62 (br s, 1H, NH), 6.75 (d, J = 8.1 Hz, 1H, aromat.), 7.05 (s, 1H, aromat.), 7.45 (d, J = 8.1 Hz, 1H, aromat.), 7.56 (br s, 1H, NH), 11.56 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 50 MHz) δ: 21.5, 42.5 (two overlapping signals), 108.8, 116.8, 120.0, 120.2, 135.6, 141.5, 152.1, 159.6. Anal. Calcd for $C_{11}H_{13}N_5$ (215.25): C, 61.38; H, 6.08; N, 32.53. Found: C, 61.12; H, 5.76; N, 32.60.

4.3.11. 7-Chloro-3-[(imidazolidin-2-yl)imino]indazole (3k)

Yield: 0.32 g (42%). White solid; mp: 223–225 °C (EtOH). IR (KBr): 3350, 3245 (NH), 3075, 2880 (CH), 1635, 1615 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 500 MHz) δ: 3.50 (br s, 4H, CH_2), 6.84 (br s, 1H, NH), 6.93–6.96 (m, 1H, aromat.), 7.34 (d, J = 7.3 Hz, 1H, aromat.), 7.46 (br s, 1H, NH), 7.57 (d, J = 7.8 Hz, 1H, aromat.), 12.25 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 42.2 (two overlapping signals), 114.8, 119.7, 120.1, 121.5, 126.2, 138.8, 153.8, 160.6. Anal. Calcd for $C_{10}H_{10}ClN_5$ (235.67): C; 50.96; H, 4.28; N, 29.72. Found: C, 50.72; H, 3.97; N, 29.98.

4.4. General procedure for the preparation of 3-[(imidazolidin-2-yl)imino]indazole hydrochlorides (4a–k)

To a suspension of the appropriate 3-[(imidazolidin-2-yl)imino]indazole (**3a–k**) (1 mmol) in anhydrous methanol (5 ml) was added dropwise HCl/MeOH solution (5.98 g/100 ml, 0.74 ml, 1.2 mmol). The cooling bath was removed, and the resulting solution or mixture was stirred at 20–22 °C for 30 min. Then the solvent was evaporated under reduced pressure to dryness. The crude product thus obtained was purified by crystallization from suitable solvent. In this manner the following compounds were obtained.

4.4.1. 3-[(Imidazolidin-2-yl)imino]indazole hydrochloride (4a)

Yield: 0.12 g (50%). White solid; mp: 196–199 °C (EtOH/Et_2O). IR (KBr): 3250, 2975, 2865 (NH, $NH^{\oplus}$, CH), 1660, 1615 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 200 MHz) δ: 3.80 (s, 4H, CH_2), 7.21 (t, 1H, aromat.), 7.44–7.59 (m, 2H, aromat.), 8.07 (d, J = 8.4 Hz, 1H, aromat.), 8.50 (s, 2H, NH), 12.37 (s, 1H, NH), 13.07 (s, 1H, $NH^{\oplus}$). Anal. Calcd for $C_{10}H_{12}ClN_5$ (237.69): C, 50.53; H, 5.09; N, 29.46. Found: C, 50.23; H, 4.78; N, 29.21.

4.4.2. 4-Bromo-3-[(imidazolidin-2-yl)imino]indazole hydrochloride (4b)

Yield: 0.13 g (41%). White solid; mp: 256–260 °C (EtOH/Et_2O). IR (KBr): 3305, 3075, 3030, 2985, 2890 (NH, $NH^{\oplus}$, CH), 1660, 1625 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 200 MHz) δ: 3.67 (s, 4H, CH_2), 7.28–7.43 (m, 2H, aromat.), 7.61 (d, J = 8.0 Hz, 1H, aromat.), 8.49 (s, 2H, NH), 10.62 (br s, 1H, NH), 13.75 (s, 1H, $NH^{\oplus}$). Anal. Calcd for $C_{10}H_{11}BrClN_5$ (316.58): C, 37.94; H, 3.50; N, 22.12. Found: C, 37.77; H, 3.34; N, 21.97.

4.4.3. 4-Chloro-3-[(imidazolidin-2-yl)imino]indazole hydrochloride (4c)

Yield: 0.12 g (43%). White solid; mp: 265–267 °C (EtOH/Et_2O). IR (KBr): 3325, 3160, 2990, 2890 (NH, $NH^{\oplus}$, CH), 1650, 1625 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 500 MHz) δ: 3.70 (s, 4H, CH_2), 7.25 (d, J = 7.3 Hz, 1H, aromat.), 7.41 (t, 1H, aromat.), 7.57 (d, J = 8.3 Hz, 1H, aromat.), 8.53 (s, 2H, NH), 10.66 (s, 1H, NH), 13.67 (s, 1H, $NH^{\oplus}$). Anal. Calcd for $C_{10}H_{11}Cl_2N_5$ (272.14): C, 44.13; H, 4.07; N, 25.73. Found: C, 43.92; H, 3.89; N, 25.48.

4.4.4. 3-[(Imidazolidin-2-yl)imino]-4-methylindazole hydrochloride (4d)

Yield: 0.12 g (50%). White solid; mp: 209–211 °C (EtOH/Et_2O). IR (KBr): 3340, 3215, 2965, 2900 (NH, $NH^{\oplus}$, CH), 1655, 1630 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 500 MHz) δ: 2.61 (s, 3H, CH_3), 3.70 (s, 4H, CH_2), 6.91 (d, J = 6.8 Hz, 1H, aromat.), 7.27–7.30 (m, 1H, aromat.), 7.37 (d, J = 8.8 Hz, 1H, aromat.), 8.48 (s, 2H, NH), 10.86 (s, 1H, NH), 13.23 (s, 1H, $NH^{\oplus}$). Anal. Calcd for $C_{11}H_{14}ClN_5$ (251.71): C, 52.49; H, 5.61; N, 27.82. Found: C, 52.19; H, 5.38; N, 27.56.

4.4.5. 3-[(Imidazolidin-2-yl)imino]-4-methoxyindazole hydrochloride (4e)

Yield: 0.14 g (52%). White solid; mp: 229–230 °C (EtOH/Et_2O). IR (KBr): 3350, 3230, 3020, 2925 (NH, $NH^{\oplus}$, CH), 1655, 1620 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 200 MHz) δ: 3.74 (s, 4H, CH_2), 3.93 (s, 3H, OCH_3), 6.55 (d, J = 7.7 Hz, 1H, aromat.), 7.03 (d, J = 8.4 Hz, 1H, aromat.), 7.32 (t, 1H, aromat.), 8.76 (s, 2H, NH), 10.45 (s, 1H, NH), 13.02 (s, 1H, $NH^{\oplus}$). Anal. Calcd for $C_{11}H_{14}ClN_5O$ (267.71): C, 49.35; H, 5.27; N, 26.16. Found: C, 49.02; H, 4.98; N, 26.08.

4.4.6. 5-Chloro-3-[(imidazolidin-2-yl)imino]indazole hydrochloride (4f)

Yield: 0.12 g (43%). White solid; mp: 253–256 °C (*i*-PrOH). IR (KBr): 3390, 3320, 3190, 2980, 2885 (NH, $NH^{\oplus}$, CH), 1660 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 500 MHz) δ: 3.75 (s, 4H, CH_2), 7.44 (d, J = 8.5 Hz, 1H, aromat.), 7.56 (d, J = 8.5 Hz, 1H, aromat.), 8.14 (s, 1H, aromat.), 8.46 (s, 2H, NH), 12.34 (br s, 1H, NH), 13.22 (s, 1H, $NH^{\oplus}$). Anal. Calcd for $C_{10}H_{11}Cl_2N_5$ (272.14): C, 44.13; H, 4.07; N, 25.73. Found: C, 43.92; H, 3.82; N, 25.78.

4.4.7. 3-[(Imidazolidin-2-yl)imino]-5-methylindazole hydrochloride (4g)

Yield: 0.10 g (42%). White solid; mp: 233–236 °C (EtOH/Et_2O). IR (KBr): 3300, 3215, 2920 (NH, $NH^{\oplus}$, CH), 1670, 1625 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 200 MHz) δ: 2.40 (s, 3H, CH_3), 3.74 (s, 4H, CH_2), 7.26 (dd, J = 8.5 Hz, J = 1.3 Hz, 1H, aromat.), 7.41 (d, J = 8.5 Hz, 1H, aromat.), 7.72 (s, 1H, aromat.), 8.42 (s, 2H, NH), 12.04 (br s, 1H, NH), 12.86 (s, 1H, $NH^{\oplus}$). Anal. Calcd for $C_{11}H_{14}ClN_5$ (251.71): C, 52.49; H, 5.61; N, 27.82. Found: C, 52.27; H, 5.32; N, 27.71.

4.4.8. 3-[(Imidazolidin-2-ylo)imino]-5-methoxyindazole hydrochloride (4h)

Yield: 0.13 g (48%). White solid; mp: 210–213 °C (EtOH/Et_2O). IR (KBr): 3390, 3310, 3190, 2910, 2830 (NH, $NH^{\oplus}$, CH), 1670, 1635 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 200 MHz) δ 3.73 (s, 4H, CH_2), 3.78 (s, 3H, OCH_3), 7.08 (dd, J = 9.1 Hz, J = 2.3 Hz, 1H, aromat.), 7.42 (d, J = 9.1 Hz, 1H, aromat.), 7.46 (d, J = 2.3 Hz, 1H, aromat.), 8.40 (s, 2H, NH), 12.14 (s, 1H, NH), 12.87 (s, 1H, $NH^{\oplus}$). Anal. Calcd for $C_{11}H_{14}ClN_5O$ (267.71): C, 49.35; H, 5.27; N, 26.16. Found: C, 49.11; H, 5.08; N, 26.32.

4.4.9. 6-Chloro-3-[(imidazolidin-2-yl)imino]indazole hydrochloride (4i)

Yield: 0.15 g (53%). White solid; mp: 309–311 °C (EtOH/Et_2O). IR (KBr): 3220, 3135, 2860, 2850 (NH, $NH^{\oplus}$, CH), 1655, 1615 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 500 MHz) δ: 3.74 (s, 4H, CH_2), 7.20 (d, J = 8.8 Hz, 1H, aromat.), 7.59 (s, 1H, aromat.), 8.05 (d, J = 8.8 Hz, 1H, aromat.), 8.46 (s, 2H, NH), 12.39 (s, 1H, NH), 13.17 (s, 1H, $NH^{\oplus}$). Anal. Calcd for $C_{10}H_{11}Cl_2N_5$ (272.14): C, 44.13; H, 4.07; N, 25.73. Found: C, 42.93; H, 3.87; N, 25.57.

4.4.10. 3-[(Imidazolidin-2-yl)imino]-6-methylindazole hydrochloride (4j)

Yield: 0.12 g (50%). White solid; mp: 223–228 °C (EtOH/Et_2O). IR (KBr): 3305, 3155, 2925, 2850 (NH, $NH^{\oplus}$, CH), 1665, 1620 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 200 MHz) δ: 2.43 (s, 3H, CH_3), 3.74 (s, 4H, CH_2), 6.99 (d, J = 8.4 Hz, 1H, aromat.), 7.27 (s, 1H, aromat.), 7.85 (d, J = 8.4 Hz, 1H, aromat.), 8.42 (s, 2H, NH), 12.16 (s, 1H, NH), 12.80 (s, 1H, $NH^{\oplus}$). Anal. Calcd for $C_{11}H_{14}ClN_5$ (251.71): C, 52.49; H, 5.61; N, 27.82. Found: C, 52.12; H, 5.42; N, 27.61.

4.4.11. 7-Chloro-3-[(imidazolidin-2-yl)imino]indazole hydrochloride (4k)

Yield: 0.12 g (43%). White solid; mp: 277–280 °C (EtOH/Et_2O). IR (KBr): 3280, 3130, 2980, 2895, 2860 (NH, $NH^{\oplus}$, CH), 1665, 1630 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6, 500 MHz) δ: 3.76 (s, 4H, CH_2), 7.17–7.20 (m, 1H, aromat.), 7.56 (d, J = 7.3 Hz, 1H, aromat.), 8.01 (d, J = 7.8 Hz, 1H, aromat.), 8.45 (s, 2H, NH), 12.34 (br s, 1H, NH), 13.48 (s, 1H, $NH^{\oplus}$). Anal. Calcd for $C_{10}H_{11}Cl_2N_5$ (272.14): C, 44.13; H, 4.07; N, 25.73. Found: C, 44.01; H, 3.92; N, 25.63.

4.5. Radioligand binding assays

4.5.1. I_1-Binding site assay

Kidneys were obtained *post mortem* from male Sprague–Dawley rats (250–280 g) and crude P_2 membranes prepared according to methods of Lione et al.[27] [^{3}H]clonidine (3 nM, Perkin–Elmer) was bound in the presence of 10 μM rauwolscine to preclude binding to α_2-adrenoceptors. The specific component was defined by 10 μM rilmenidine; under these conditions, the site labelled represents a model of the central I_1 binding site.[28] Membrane aliquots (400 μl, 0.2–0.5 mg protein) were incubated with 11 concentrations of the test compounds over the range 0.01 μM–100 mM. Incubations were carried out in 50 mM Tris–HCl buffer (pH 7.4) at room temperature for 45 min. Bound radioligand and free radioactivity were separated by rapid filtration through pre-soaked (0.5% polyethyleneimine) glass-fibre filters (Whatman GFB). Trapped radioligand was determined by liquid scintillation counting and the data were analysed with GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA, USA) to yield IC_{50} values (the concentration of tested ligand that displaces 50% of specifically bound [^{3}H]clonidine).

4.5.2. α_1-, α_2- and I_2-Binding site assays

Brains were obtained *post mortem* from male Sprague–Dawley rats (250–280 g) and crude P_2 membranes prepared according to methods of Lione et al.[27] Membrane aliquots (400 μl, 0.2–0.3 mg protein) were incubated with 11 concentrations of the tested compounds over the range 0.01 nM–100 μM in the presence of the selective I_2 binding site ligand [^{3}H]2BFI[28] (1 nM), the α_1-adrenoceptor antagonist [^{3}H]prazosin (1 nM) or the α_2-adrenoceptor antagonist [^{3}H]RX821002[29] (1 nM) in a final volume of 500 μl. Non-specific binding was determined using 10 μM BU224[30] for defining I_2 binding, 10 μM phenylephrine for α_1-adrenoceptors and 10 μM rauwolscine to define α_2-adrenoceptor binding. Each incubation was performed in triplicate at room temperature and was allowed to reach equilibrium (45 min). Bound and free radioactivity were separated by rapid filtration through pre-soaked (0.5% polyethyleneimine) glass-fibre filters (Whatman GF/B). Filters were then washed twice with 5 ml of ice-cold buffer and membrane-bound radioactivity remaining on the filters was determined by liquid scintillation counting. Data were analysed by iterative non-linear regression curve fitting procedures with GraphPad Prism. Each experiment was analysed individually and equilibrium dissociation constants (K_i) were determined by the method of Cheng and Prusoff.[22] The resulting values are given as means of three or four separate experiments.

4.6. Estimation of agonist potency and efficacy

4.6.1. Cell culture and transfections

Adherent Chinese hamster ovary (CHO) cells (K1 strain) (American Type Culture Collection, Manassas, VA, USA), transfected to express the human α_{2A}-adrenoceptor subtype,[23] were cultured in α-minimum essential medium supplemented with 2 mM glutamine, 20 mM $NaHCO_3$, 5% heat-inactivated foetal calf serum, penicillin (50 units ml^{-1}) and streptomycin (50 μg ml^{-1}). The pc DNA3.1+-based expression constructs were originally transfected into CHO cells using the Lipofectamine™ 2000 reagent kit (Invitrogen Life Technologies, Inc., Rockville, MD, USA). Stable transfections were selected using 800 μg ml^{-1} of the neomycin analogue G418 (Calbiochem, San Diego, CA, USA). After selection, transfected cell cultures were examined for their ability to bind the α_2-adrenoceptor antagonist radioligand [^{3}H]RX821002. The cells were subsequently maintained in 200 μg ml^{-1} G418. Confluent cells were harvested into chilled phosphate-buffered saline, pelleted and frozen at −70 °C.

4.6.2. Membrane preparation

All procedures were performed on ice. The harvested recombinant CHO cell pellets were thawed and suspended in hypotonic lysis buffer (10 mM Tris–HCl, 0.1 mM EDTA, 0.32 mM sucrose, pH 7.4) and homogenised using an Ultra-Turrax homogeniser (3 × 10 s at 800 rpm). The homogenate was centrifuged at 23,000*g* for 30 min, and the pellet was re-homogenised and again centrifuged at 23,000*g* for 30 min. The membrane pellet was suspended in hypotonic lysis buffer and stored at −70 °C until used. Protein concentrations were determined with the method of Bradford[24] using bovine serum albumin as reference.

4.6.3. [^{35}S]GTPγS Binding assay

Agonist-induced stimulation of [^{35}S]GTPγS binding was measured essentially as described previously.[25] Briefly, membranes were thawed and diluted with binding buffer (50 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl, 10 μM GDP, 1 mM DTT, 30 μM ascorbic acid, pH 7.4). Incubations were performed on 96-well Millipore MultiScreen MAFB glass-fibre filter plates (Millipore Corp., Bedford, MA, USA). Samples containing 5 μg of membrane protein were preincubated with agonists for 30 min at 37 °C prior to addition of 0.1 nM [^{35}S]GTPγS. Reactions were terminated after 60 min incubation at 37 °C by vacuum filtration using a Millipore MultiScreen Vacuum Manifold. The filter plates were washed three times with cold wash buffer (20 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, pH 7.4). Filters were dried, and 50 μl SuperMix scintillation cocktail was added into each well. The incorporated radioactivity was measured using a MicroBeta scintillation counter (Perkin–Elmer Wallac, Turku, Finland). All experiments were performed in duplicate and repeated at least three times. Analysis of the results with GraphPadPrism yielded estimates of agonist potency (EC_{50}) and efficacy (intrinsic activity in comparison to the natural full agonist noradrenaline).

4.6.4. In vivo studies mean arterial blood pressure (MAP) and heart rate (HR) in rats

Male Wistar rats, weighing 200–250 g, were purchased from the Animal House of the Medical University of Gdańsk, Poland. All experiments were approved by the Local Ethical Committee on Animal Experiments. The animals were fed a commercial rodent chow (Labofeed-B, Poland). Tap water was available ad libitum. Rats were anaesthetized by ip injection of thiopental (Sandoz, Austria) at the dose 70 mg/kg body weight and maintained under anaesthesia by thiopental supplementation (30 μg/kg/min) during the experiment. The animals were placed on a heated table, and body temperature was maintained between 36 and 37 °C. Tracheostomy was performed. Catheters were inserted into the carotid artery for blood pressure and heart rate monitoring, into a jugular vein for infusions, and into the bladder for free diuresis. After all surgical procedures, a 40 min recovery period was allowed to establish steady state. The rats were infused with isotonic saline (Fresenius Kabi, Poland) supplemented with thiopental at the rate of 1.2 ml/h. After 40 min of saline infusion, the tested compound was administered as a 100 μl bolus through the venous catheter doses of 0.01, 0.1 and 1.0 mg/kg. Arterial blood pressure and heart rate were monitored directly and sampled continuously at 100 Hz, as described previously,[26] using Biopac Systems, Inc., Model MP 100 (Goleta, CA, USA). The results of measurements were elaborated with the help of the ACQKnowledge (Goleta, CA, USA) measurement system and were selected, scaled and filtered to remove accidental signal disturbances. The recorded time domain transient data are presented as graphs with the help of Excel (Microsoft, USA).

ANOVA was performed for ΔMAP and ΔHR, calculated as the difference in MAP and in HR from baseline measurements ('time 0') for each group, as described previously.[26] This allowed for direct comparison of responses to treatment between groups. Data were analyzed by ANOVA with repeated measurements, using Statistica StatSoft software (StatSoft, Inc., Tulsa, OK, USA), after test compound or vehicle administration. When the effect was significant, post hoc comparisons were performed using Fisher's test. A value of $p < 0.05$ was considered statistically significant.

4.7. X-ray structure analysis

Crystal data for $C_{10}H_{11}N_5$ (**3a**): monoclinic, space group $P2_1/n$, $a = 8.1911(3)$, $b = 10.0631(2)$, $c = 12.2263(4)$ Å, $\beta = 108.896(4)^\circ$, $V = 953.48(5)$ Å^3, $Z = 4$, $d_x = 1.403$ g cm^{-3}, $T = 293$ K. Data were collected for a crystal with dimensions $0.3 \times 0.3 \times 0.5$ mm^3 with a KumaCCD diffractometer using graphite monochromated Mo Kα radiation. Final R indices for 1762 reflections with $I > 2\sigma(I)$ and 149 refined parameters are: $R_1 = 0.0335$, $wR_2 = 0.0878$ ($R_1 = 0.0455$, $wR_2 = 0.0983$ for all 2258 data). Atom labelling is shown in Figure 1.

Tables of atomic coordinates, bond lengths, and bond angles have been deposited with the Cambridge Crystallographic Data Centre (the deposition No. CCDC 776303). These tables may be obtained on request from the Director, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.11.020. These data include MOL files and InChiKeys of the most important compounds described in this article.

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