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Inhibition of Neuronal Nitric Oxide Synthase by 7-Methoxyindazole and Related Substituted Indazoles

Pascale Schumann,[a] Valérie Collot,[b] Yannick Hommet,[a] Willy Gsell,[a] François Dauphin,[a] Jana Sopkova,[b] Eric T. MacKenzie,[a] Dominique Duval,[a] Michel Boulouard[b] and Sylvain Rault[b,*]

[a]*CNRS UMR 6551, Université de Caen, Bd H Becquerel, 14074 Caen, France*
[b]*UPRES-EA 2126, Centre d'Etudes et de Recherches sur le Médicament de Normandie (CERMN) - UFR Sciences Pharmaceutiques, rue Vaubénard, Caen, France*

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Abstract—The synthesis and pharmacological evaluation of methoxyindazoles as new inhibitors of neuronal nitric oxide synthase are presented. The 7-methoxyindazole, although less potent than 7-NI, is the most active compound of the series in an in vitro enzymatic assay of neuronal nitric oxide synthase activity. This result shows that the nitro-substitution is not indispensable to the biological activity of the indazole ring. 7-Methoxyindazole possesses in vivo NOS inhibitory as well and related antinociceptive properties.

Nitric oxide (NO) is an important biological messenger involved in numerous physiological processes, including neurotransmission, blood pressure and blood flow regulation, platelet aggregation and inflammation (for review, see ref 1). On the other hand, overproduction of NO plays a role in a variety of disorders, such as septic shock, pain, ischaemia and several neurodegenerative diseases.[2] NO is synthesised in several cell types from L-arginine by different isoforms of nitric oxide synthase (NOS). To date, three isoforms have been cloned: neuronal (nNOS) and endothelial (eNOS) types, which are both constitutive and calcium dependent and an inducible, calcium independent one (iNOS).[3] Since these isoforms possess a distinct cellular localisation and are differentially regulated, they represent specific targets for potential therapeutical approaches. Development of inhibitors selective of one of these isoforms is therefore of considerable interest, both for a therapeutic purpose and for their use as specific pharmacological tools. For example, NO of neuronal origin is involved in pain transmission[4,5] and constitutes, thus, a potential target for antinociceptive drugs. However, such a drug needs to be selective for nNOS, i.e., leaving the eNOS unaffected, which would otherwise lead to hypotensive side effects. Though there is a quite low homology between the three human NOS primary sequences (approximately 50%), the active site of the enzymes seems, however, to be relatively conserved,[6] presumably explaining the difficulty to obtain selective inhibitors. Indeed, several nNOS inhibitors have been developed over the last decade but only few present both a potency and a clear selectivity toward this isoform. The first inhibitors developed belong to the L-arginine analogues family[7] and are mostly not selective for the neuronal isoform. Another series of inhibitors is constituted by heterocycles such as substituted indazole and imidazole. To date, only the 1-(2-trifluoromethylphenyl) imidazole (TRIM) has been reported to be relatively selective for nNOS in comparison to eNOS but its potency is rather weak ($IC_{50} = 30\,\mu M$ vs nNOS).[8] The nitroindazole family (with 7-nitroindazole, 7-NI, as the lead compound) are greater nNOS inhibitors but their selectivity over the other isoforms remain low, at least in vitro.[4,9] The aim of this study was to pharmacomodulate the indazole nucleus with electron-donating substituents in order to develop novel potent and specific inhibitors of nNOS.

Chemistry

Most of the syntheses of the indazole derivatives reported in the literature proceed from benzene precursors in which the pyrazole moiety was generated by ring closure

*Corresponding author. Fax: +33-2-31-93-11-88; e-mail: rault@pharmacie.unicaen.fr

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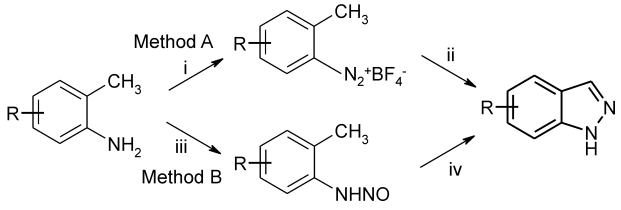
starting from isatines, phenylhydrazines or *o*-toluidines.[10–12] Amongst these reactions, we found that the most common proceed by a phase transfer catalysed reaction from *o*-methylbenzendiazonium tetrafluoroborates (Method A),[13] except for compounds **6** and **8** where the cyclisation of *N*-nitroso derivatives[14] was found to be superior (Method B) as presented in Scheme 1.

Indazoles bearing either electron-withdrawing or electron-donating substituents may be prepared by these routes (Table 1).

We obtained 3-iodo-7-methoxyindazole **9** starting from **8**, with a 90% yield by modifying[15] the Bocchi method[16] (Scheme 2).

On the other hand, treatment of **8** with boron tribromide produced suitable 7-hydroxyindazole **10**[17] for further functionalisation. Then, we found that treatment of **10** with either ethyl iodide or benzyl bromide in the presence of potassium carbonate gave a regioselectively to the *O*-alkylated derivatives **11a**–**b** without any traces of N1 or N2 substituted compounds.[18]



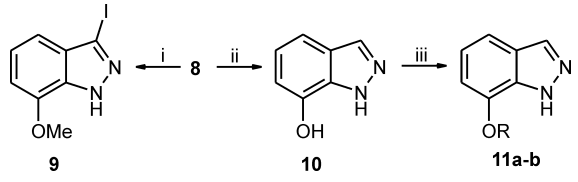
Scheme 1. Synthesis of 1-*H*-indazoles. Reagents and conditions: (i) (1) HBF_4 aq 50%; (2) $NaNO_2$ aq, 0 °C; (ii) AcOK, 18-crown-6, $CHCl_3$, rt; (iii) AcOH, HCl concd, $NaNO_2$; (iv) benzene, reflux.

Table 1. Formation of 1-*H*-indazoles (Method A[13] or B[14])

	R	Method	Yield (%)
1	5-Br	A	63
2	5-F	A	65
3	6-Cl	A	90
4	6-CH_3	A	55
5	4-OCH_3	A	22
6	5-OCH_3	B	60
7	6-OCH_3	A	38
8	7-OCH_3	B	80

Biological Evaluation

Effects of the derivatives (among them **5**,[19] **9**,[20] **10**,[17] **11a**–**b**[18] are new compounds) on nNOS activity were evaluated in vitro on rat cerebellum homogenates. IC_{50}'s were determined from the NOS inhibition curves constructed with four concentrations (0.1, 1, 10 and 100 μM). Enzymatic activities were assayed by monitoring the conversion of L-[^{3}H] arginine to L-[^{3}H] citrulline according to a previously described method[7] (1 mM $CaCl_2$, 200 μM β-NADPH, 0.88 μM L-arginine, 0.12 μM L-[^{3}H] arginine, 15 min at 37 °C). Basal activity represented 120 ± 15 pmol citrulline formed/mg protein/h and the already described lead compound, 7-NI (dissolved in DMSO 0.4%) exhibited an IC_{50} value of 0.8 ± 0.1 μM (mean ± SEM; $n = 3$ experiments). Since the IC_{50} values of the first four indazole derivatives were relatively high (75 μM, > 100 μM, 28 μM, > 100μM for, respectively, 5-bromoindazole, 5-fluoroindazole, 6-chloroindazole, 6-methylindazole), we focused on the methoxyindazole series. As shown in Table 2, the most potent compound in this series was 7-methoxyindazole (7-MI with an IC_{50} value of 6.3 ± 2.9 μM ($n = 6$). This result confirms the importance of the position 7 on the indazole nucleus, as already suggested.[9] Pharmacomodulation of the indazole nucleus had been performed earlier[9,21] and it had been concluded that substitution with electron-withdrawing substituents (such as nitro or halogens) increases the NOS inhibitory potency while electron-donating substituents would decrease this ability. This is not the case in our experiments since an electron-donating substituent such as a methoxy group rather increases the inhibitory effect of the indazole (more than 50% inhibition of nNOS for 7-MI vs 11% inhibition for indazole, both at 10 μM). However, other factors, such as the size of the substituent, could also influence the biological activity of 7-substituted indazoles. Addition of a halogen (iodine) on position 3 of 7-MI did not improve but rather abolished its activity (Table 2). This result is quite surprising since it had been



Scheme 2. Synthesis of 7-substituted indazoles **10** and **11**. Reagents and conditions: (i) I_2, KOH, DMF, rt; (ii) BBr_3, CH_2Cl_2, Δ; (iii) C_2H_5I or BnBr, K_2CO_3, acetone, Δ.

Table 2. IC_{50} values of the methoxyindazoles and related substituted indazoles against rat cerebellar nNOS

Compound	IC_{50}, μM mean ± SEM	% Inhibition at 100 μM	Vehicle	Number of experiments
7-Methoxyindazole	6.3 ± 2.9	80%	Ethanol 0.4%	6
6-Methoxyindazole	No effect	0	Ethanol 0.4%	1
5-Methoxyindazole	> 100	25%	DMSO 0.4%	1
4-Methoxyindazole	> 100	30%	DMSO 0.8%	1
3-Iodo-7-methoxy indazole	No effect	0	DMSO 0.4%	2
7-Hydroxyindazole	> 100	7%	DMSO 0.4%	1
7-Ethoxyindazole	100	50%	DMSO 0.4%	1
7-Benzyloxyindazole	No effect	0	DMSO 0.4%	1

Table 3. Antinociceptive activity of 7-methoxyindazole, 7-nitroindazole, and acetylsalicylic acid in the writhing test in mice ($n=8$ per group)

Compound	Dose (mg/kg)	Number of writhes (mean ± SEM)
Arachis oil		21.2 ± 1.0
7-Methoxyindazole	10	15.4 ± 1.8[a]
	25	12.2 ± 2.2[a]
	50	3.7 ± 1.2[b]
7-Nitroindazole	50	6.2 ± 1.6[b]
Acetylsalicylic acid	15	3.5 ± 0.6[b]

[a] $P<0.05$.
[b] $P<0.001$ versus control (arachis oil) (ANOVA and PLSD of Fischer).

Table 4. Effect of L-arginine pre-treatment on antinociceptive activity of 7-methoxyindazole ($n=8$ per group)

Compound	Dose (mg/kg)	Number of writhes (mean ± SEM)
Arachis oil		23.8 ± 1.6
L-Arginine	50	20.8 ± 1.7
7-Methoxyindazole	50	4.7 ± 1.2[b]
L-Arginine	50	
+ 7-Methoxyindazole	50	10.6 ± 1.2[a,c]

[a] $P<0.05$.
[b] $P<0.001$ versus control.
[c] $P<0.05$ versus 7-methoxyindazole (ANOVA and PLSD of Fischer).

reported that addition of a halogen (bromine) substantially increased the inhibitory properties of 7-NI.[21] The biological effect of 7-MI seems to be directly linked to its peculiar structure because neither hydroxy nor ethoxy or benzyloxy were able to produce NOS inhibition (Table 2).

In order to characterise the in vivo pharmacology of 7-MI, the drug was administered intraperitoneally in rats (50 mg/kg, dissolved in arachis oil, $n=6$) and the cerebellum nNOS activity, according to the previously described method, was determined ex vivo 30 min later. 7-MI induced an inhibition of nNOS of 42 ± 7% which is lower than 7-NI tested in the same conditions (92 ± 1%, $n=3$)[22] but comparable to TRIM (45 ± 3%, 50 mg/kg saline, $n=2$, data not shown). In vivo inhibitory effects of 7-MI on nNOS were further analysed on a mouse model of nociception, the writhing test.[23] 7-MI (10, 25, 50 mg/kg, dissolved in arachis oil) and acetylsalicylic acid (15 mg/kg) were administered ip, 15 min before an ip injection of 0.6% acetic acid solution and the number of writhes was counted during 10 min, 10 min after acetic acid administration (Table 3).

In these conditions, 7-MI produced a dose-dependent antinociception with, at the higher dose, a maximal effect close to that of acetylsalicylic acid (15 mg/kg) and slightly more important than that of 7-NI (50 mg/kg, ip).The antinociceptive effect of 7-MI (50 mg/kg, ip) was partially reversed by pretreatment with L-arginine (50 mg/kg, ip administered 5 min before 7-MI) (Table 4).

As an initial attempt to evaluate the selectivity of 7-MI toward nNOS versus the other isoforms, the effects of this compound on systemic arterial pressure were determined in the conscious rat. At the dose of 50 mg/kg i.p., 7-MI did not modify mean arterial blood pressure 30 min as well as 1 h after injection (116 ± 4 mmHg before vs 111 ± 5 mmHg 30 min after and 114 ± 5 mmHg 60 min after administration, $n=6$, not significantly different, ANOVA). However, since 7-NI, in the same conditions (50 mg/kg, ip) did not alter mean arterial pressure (117 ± 2 mmHg before vs 113 ± 5 mmHg 30 min later, $n=6$), these results need to be interpreted with caution in terms of selectivity. Indeed, the effects of 7-NI on eNOS are rather controversial. Although in vitro data show that this compound inhibits bovine eNOS with an IC_{50} comparable to the IC_{50} against nNOS,[9] the lack of effects of 7-NI on systemic arterial pressure would suggest an absence of in vivo inhibition of rat eNOS. Thereafter, it was proposed that the absence of cardiovascular changes after 7-NI administration was due to the use of anaesthetics with cardiovascular depressive effects.[24] However, our results do not support this hypothesis since, in the conscious animal, 7-NI failed to increase, even slightly, arterial blood pressure. As neither 7-NI nor 7-MI modified arterial pressure under our conditions, the comparison of both drugs in term of specificity is rendered impossible. The selectivity of 7-MI against the three isoforms will have to be further analysed on adapted in vitro enzymatic assays.

Conclusion

We have reported the synthesis and pharmacological evaluation of methoxyindazoles. 7-MI, the most active compound of the series is a novel lead in the field of NOS inhibitory drugs development. This result shows that the nitro-substitution is not indispensable to the biological activity of the indazole ring. It argues against the idea that the potency of 7-nitroindazole is due to its electronic withdrawing effect. We currently aim to compare the interactions of 7-MI and 7-NI with the enzyme NOS by modelisation, based on the crystallographic structure of 7-NI,[25] 7-MI and of the NOS oxygenase domain.[26]

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17. 7-Hydroxyindazole (**10**): Boron tribromide (1.0 M solution in CH_2Cl_2) (32.4 mL, 32.4 mmol) was added dropwise to a cold solution of 7-methoxyindazole **8** (1.80 g, 12.1 mmol) in CH_2Cl_2 (30 mL). The reaction mixture was stirred and heated to reflux. After 4 h, the solution was cooled to 0 °C. Water (7.4 mL) was added dropwise then 10% aqueous $NaHCO_3$ until pH 7–8. The reaction mixture was extracted with EtOAc (3×50 mL). The combined organic layers were dried ($MgSO_4$), filtered and evaporated in vacuo. The residue was purified by flash chromatography on silica gel (acetone/cyclohexane, 3:7) to afford **10** (1.30 g, 80%) as a beige solid: mp 179 °C (toluene); ^{1}H NMR (DMSO-d_6) δ 6.64 (d, 1H, J=8.1 Hz), 6.87 (t, 1H, J=8.1 Hz), 7.14 (d, 1H, J=8.1 Hz), 7.94 (s, 1H), 10.02 (br s, 1H), 12.99 (br s, 1H).
18. 7-Ethoxyindazole (**11a**): To a solution of **10** (0.30 g, 2.24 mmol) in acetone (8 mL) was added iodoethane (0.22 mL, 2.24 mmol) and potassium carbonate (1.55 g, 11.20 mmol). The mixture was heated 3 h to reflux, and then the solvent was removed in vacuo. The residue was dissolved in EtOAc (30 mL), washed with H_2O (2×15 mL), dried ($MgSO_4$), filtered and evaporated. The solid residue was purified by flash chromatography on silica gel (EtOAc/cyclohexane, 1:8) to afford **11a** (0.25 g, 68%) as a white solid: mp 112 °C; IR (KBr) 3155, 2918, 1590, 1261, 953, 724 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.34 (t, 3H, J=7.2 Hz), 4.12 (q, 2H, J=7.2 Hz), 6.64 (d, 2H, J=7.5 Hz), 6.98 (t, 1H, J=7.5 Hz), 7.24 (d, 1H, J=7.9 Hz), 8.00 (s, 1H), 10.92 (br s, 1H). 7-Benzyloxyindazole (**11b**): As above, starting from **10** (0.18 g, 1.34 mmol) in solution in acetone (8 mL) was added benzyl bromide (0.15 mL, 1.34 mmol) and potassium carbonate (0.93 g, 6.70 mmol). The residue was purified by flash chromatography on silica gel (EtOAc/cyclohexane, 1:9) to afford **11b** (0.22 g, 72%) as a pink solid: mp 108 °C; IR (KBr) 3160, 2912, 1588, 1261, 951, 727 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 5.23 (s, 2H), 6.81 (d, 1H, J=7.6 Hz), 7.08 (t, 1H, J=7.7 Hz), 7.29–7.49 (m, 6H), 7.99 (s, 1H), 10.73 (br s, 1H).
19. 4-Methoxyindazole (**5**) (Method A): A cooled aqueous solution of sodium nitrite (1.22 g in 2.45 mL H_2O, 1.79 mmol) was added at 0 °C dropwise to a ice cooled solution of 3-methoxy-2-methylaniline (2.45 g, 17.9 mmol) dissolved in fluoroboric acid (50% solution in water; 7.35 mL). After the end of the addition, the mixture was stirred 1 h without cooling. The resulting precipitate was filtered and washed with Et_2O (3×100 mL) to obtain 4-methoxy-2-methylphenyldiazonium tetrafluoroborate salt as a pink solid (4.05 g, 96%): mp 150 °C; IR (KBr): 3426, 2231, 1590, 1062–1027 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.65 (s, 3H), 3.95 (s, 3H), 7.72 (t, 1H, J=9.0 Hz), 7.80 (d, 1H, J=9.0 Hz), 8.17 (d, 1H, J=9.0 Hz). The diazonium tetrafluoroborate salt (1 g, 4.23 mmol) was added under nitrogen in one portion to a stirred mixture of dried and powdered potassium acetate (0.85 g, 8.46 mmol) and 18-crown-6 (0.05 g, 0.21 mmol) in chloroform (40 mL). After 2 h, the resulting precipitate was filtered, washed with H_2O (3×50 mL), dried over $CaCl_2$ and evaporated in vacuo. The crude gum was recrystallised in boiling H_2O and filtered to obtain a white solid (0.070 g, 11%): mp 118 °C; IR (KBr): 3117, 2941, 1593, 1254, 983, 735 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.89 (s, 3H), 6.52 (d, 1H, J=7.8 Hz), 7.07 (d, 1H, J=7.8 Hz), 7.23 (t, 1H, J=7.8 Hz), 8.00 (s, 1H), 13.00 (br s, 1H).
20. 3-Iodo-7-methoxyindazole (**9**): A solution of iodine (4.45 g, 17.54 mmol) and potassium hydroxide pellets (1.85 g, 32.89 mmol) were successively added into a DMF solution (25 mL) of 7-methoxyindazole **8** (1.30 g, 8.77 mmol) at room temperature under stirring. After 1 h, the reaction mixture was poured into 10% aqueous $NaHSO_3$ (100 mL) and extracted with Et_2O (3×100 mL). The combined organic layers were washed with water and brine, dried ($MgSO_4$), and the solvent was evaporated. The residue was purified by flash chromatography on silica gel (EtOAc/cyclohexane, 1:5) to afford **9** (1.80 g, 75%) as a beige solid: mp 140 °C (toluene); IR (KBr) 3334, 1516, 1313, 1258, 1004, 775 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.98 (s, 3H), 6.79 (d, 1H, J=8.0 Hz), 7.08 (d, 1H, J=8.0 Hz), 7.14 (t, 1H, J=8.0 Hz), 10.82 (br s, 1H).
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