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Synthesis, Pharmacological Study and Modeling of 7-Methoxyindazole and Related Substituted Indazoles as Neuronal Nitric Oxide Synthase Inhibitors

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The synthesis, pharmacological evaluation and modelisation of 7-methoxyindazole (7-MI) and related alkoxyindazoles as novel inhibitors of neuronal nitric oxide synthase are presented. 7-MI remains the most active compound of this series in an *in vitro* enzymatic assay of neuronal nitric oxide synthase activity. Modeling studies of the interaction of 7-substituted indazole derivatives complexed with nNOS and the relationship with their respective biological activities suggest that a bulky substitution on position-7 is responsible for a steric hindrance effect which does not allow these compounds to interact with nNOS in the same way as 7-NI and 7-MI.

Keywords: 7-Methoxyindazole; Neuronal nitric oxide synthase; Inhibitors; Molecular modelling; Structure–activity relationships

INTRODUCTION

Nitric oxide (NO) is an important biological messenger involved in numerous physiological processes, including neurotransmission, blood pressure and blood flow regulation, platelet aggregation, inflammation (for review, see reference[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 form (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 to one of these isoforms is therefore of considerable interest, both for a therapeutical purpose and for their use as specific pharmacological tools. For example, NO of neuronal origin is involved in pain transmission[4,5] and thus constitutes 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. The three human NOS isoforms share approximately 50% sequence identity and the active site of the enzymes is remarkably conserved,[6] presumably explaining the difficulty in obtaining selective inhibitors. Indeed, several nNOS inhibitors have been developed over the past decade but only a few present both 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. The nitro-indazole family (with 7-nitroindazole, 7-NI, as the lead compound) are more potent nNOS inhibitors but their selectivity over the other isoforms remain low, at least *in vitro*.[4,8] We recently published the pharmacomodulation of the indazole nucleus with electron-donating substituents in order to develop novel potent and specific inhibitors of nNOS.[9] The 7-methoxyindazole (7-MI with an IC_{50} value of $6.3 \pm 2.9\ \mu M$ ($n = 6$)) was the most active compound of the series in an *in vitro* enzymatic assay of neuronal

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nitric oxide synthase activity. This result confirmed the importance of position-7 on the indazole nucleus, as already suggested[8] and showed that 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 *versus* 11% inhibition for indazole, both at 10 μM).

In the present study, chemical modifications of the structure of 7-MI have been carried out in order to specify structure-activity relationships and to understand the part played by the methoxy substituent and the steric hindrance tolerated in this position. Moreover, modelisation studies of 7-substituted indazole derivatives complexed with nNOS and comparison with their respective biological activities are reported.

MATERIALS AND METHODS

Chemistry

Commercial reagents were used as received without additional purification. Melting points were determined on a Kofler melting point apparatus and are uncorrected. IR spectra were determined on a Genesis Series FTIR spectrometer. ^{1}H NMR (400 MHz) were recorded on a JEOL Lambda 400 Spectrometer. Chemical shifts are expressed in parts per million downfield from tetramethylsilane as an internal standard. Thin-layer chromatography (TLC) was performed on 0.2 mm precoated plates of silica gel 60F-264 (Merck) with visualization with ultraviolet light. Column chromatography was carried out using silica gel 60 (0.063–0.2 mm) (Merck).

7-Hydroxyindazole (2)

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 **1** (1.80 g, 12.1 mmol) in CH_2Cl_2 (30 mL) and 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 **2** (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); MS (m/z): M^+: 134 (100).

Typical Procedure for the Preparation of 3a–3d

To a solution of **2** (0.30 g, 2.24 mmol) in acetone (8 mL) was added alkyl halide (2.24 mmol, 1 eq) and potassium carbonate (1.55 g, 11.20 mmol, 5 eq). The mixture was heated to reflux for 3 h, 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).

7-Ethoxyindazole (**3a**)

(0.25 g, 68%); 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, 1H, 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); MS (m/z): M^+: 162 (100).

7-Propoxyindazole (**3b**)

(0.26 g, 67%); pink solid: mp 102°C; IR (KBr) 3157, 2957, 1590, 1263, 948, 728 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.10 (t, 3H, J = 7.2 Hz), 1.90 (m, 2H), 4.11 (t, 2H, J = 7.2 Hz), 6.71 (d, 1H, J = 7.5 Hz), 7.06 (t, 1H, J = 7.5 Hz), 7.28 (d, 1H,J = 7.9 Hz), 8.04 (s, 1H), 10.25 (br s, 1H); MS (m/z): M^+: 176 (100).

7-Butoxyindazole (**3c**)

(0.31 g, 72%); pink solid: mp 96°C; IR (KBr) 3156, 2920, 1589, 1260, 950, 728 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.98 (t, 3H, J = 7.2 Hz), 1.53 (sext, 2H, J = 7.2 Hz), 1.87 (quint, 2H, J = 7.2 Hz), 4.14 (t, 2H, J = 7.2 Hz), 6.71 (d, 2H, J = 7.5 Hz), 7.04 (t, 1H, J = 7.5 Hz), 7.30 (d, 1H, J = 7.9 Hz), 8.09 (s, 1H), 11.42 (br s, 1H);; MS (m/z): M^+: 190 (100).

7-Benzyloxyindazole (**3d**)

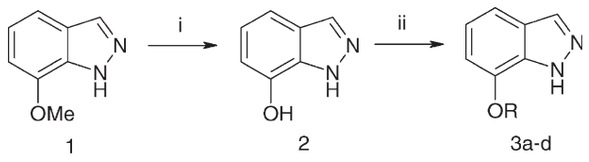
(0.37 g, 72%); pink solid: mp 108°C; IR (KBr) 3160, 2912, 1588, 1261, 951, 727 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 5.23 (s, 2H), 6.81 (d, 1H, J = 7.6 Hz), 7.08 (t, 1H, J = 7.7 Hz), 7.29-7.49 (m, 6H), 7.99 (s, 1H), 10.73 (br s, 1H);; MS (m/z): M^+: 224 (100).

Pharmacology

Inhibition of nNOS activity by the compounds was evaluated *in vitro* on rat cerebellum homogenates. IC_{50} values 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; $n = 3$).

3D Model of nNOS

The sequence of the heme domain of nNOS have been aligned with the sequences of the heme domain



	R	Yield (%)
2	H	80
3a	C_2H_5	88
3b	C_3H_7	67
3c	C_4H_9	72
3d	$C_6H_5CH_2$	89

SCHEME 1 Synthesis of 7-substituted indazoles **2**–**3**. Reagents and conditions: (i) BBr_3, I or CH_2CL_2, Δ; (ii) C_2H_2I or C_3H_7I, C_4H_9I or BnBr K_2CO_3, acetone, Δ.

of eNOS and iNOS. nNOS shares 58% and 54% sequences identity with eNOS and iNOS respectively. The structure of the dimer of the bovine eNOS catalytic domain complexed with 3-bromo-7-nitroindazole[10] (PDB entry: 1D0C) has been used as a support for the model of rat nNOS. We have used the web interface Model Tool Server[11] for the construction of the model. All molecular-mechanics calculations were performed using CHARMm[12] with potential function parameter set 22 through Accelrys software (San Diego, USA). To derive 3D models as well as parameters for the nNOS inhibitors, *ab initio* quantum-chemical calculations were performed on isolated molecules using the MOPAC program, AM1 method with full geometry optimisation combined with grid search of the stable conformation.[13] The 3D models of 7-NI and 7-MI were based on their solved crystals structures.[14,15]

The inhibitors were introduced in the active site cavity by graphical manipulation in an analogous position to that of 7-NI. These initials models were subjected to energy-minimisation. During all calculations all atoms that lay 10 Å or further from any inhibitor atom were fixed and water molecules determined in the X-ray structure of eNOS/3-Br-7-NI were included. The initial models were energy minimised to a root-mean-square energy gradient of less 0.001 kcal/Á.

RESULTS

Chemistry

7-Methoxyindazole **1** was obtained in 80% yield by ring closure of the N-nitroso derivative starting from 2-methoxy-6-methylaniline.[16] Treatment of **1** with boron tribromide suitably produced 7-hydroxyindazole **2** for further functionalisation (Scheme 1). Then, it was found that treatment of **2** with either ethyl iodide, propyl iodide, butyl iodide or benzyl bromide in the presence of potassium carbonate gave regioselectively to the O-alkylated derivatives **3a**–**d** without any traces of N1 or N2 substituted compounds.

Inhibition of nNOS Activity

The potency of the indazole derivatives against the rat cerebellar nNOS is listed in Table I. Increasing the side chain of the substituent did not improve, infact it rather decreased, the biological activity of the 7-substituted indazoles. The biological effect of 7-MI **2** seems to be directly linked to its unique structure since neither hydroxy nor ethoxy, propoxy, butoxy or benzyloxy were able to produce NOS inhibition (Table I).

Modelisation Studies

The aim of the modelling study was to try to account for the difference between the pharmacological results of 7-MI and the other alkoxyindazoles. For this purpose, several 3D models of nNOS complexed with indazoles **2**–**3** were constructed and compared with their experimentally measured activities.

The solved X-ray structures of the catalytic domains of bovine eNOS[17] and murine iNOS[18] with L-arginine have shown that the substrate guanidino side chain interacts with a glutamic acid residue in the enzyme binding site, which is remarkably conserved through all three isoforms (E363 in bovine eNOS, E371 in murine iNOS, E592 in rat nNOS). The terminal guanidinium nitrogen is located approximately 4.0 Å from the heme iron and is therefore strategically positioned for hydroxylation by the iron-bound oxygen. Only the dimeric

TABLE I IC_{50} values of 7-methoxyindazole and related substituted indazoles against rat cerebellum 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
7-hydroxyindazole	>100	7%	DMSO 0.4	1
7-ethoxyindazole	100	50%	DMSO 0.4	1
7-propoxyindazole	>100	11%	DMSO 0.4	1
7-butoxyindazole	No effect	0	Ethanol 0.4	1
7-benzyloxyindazole	No effect	0	DMSO 0.4	1

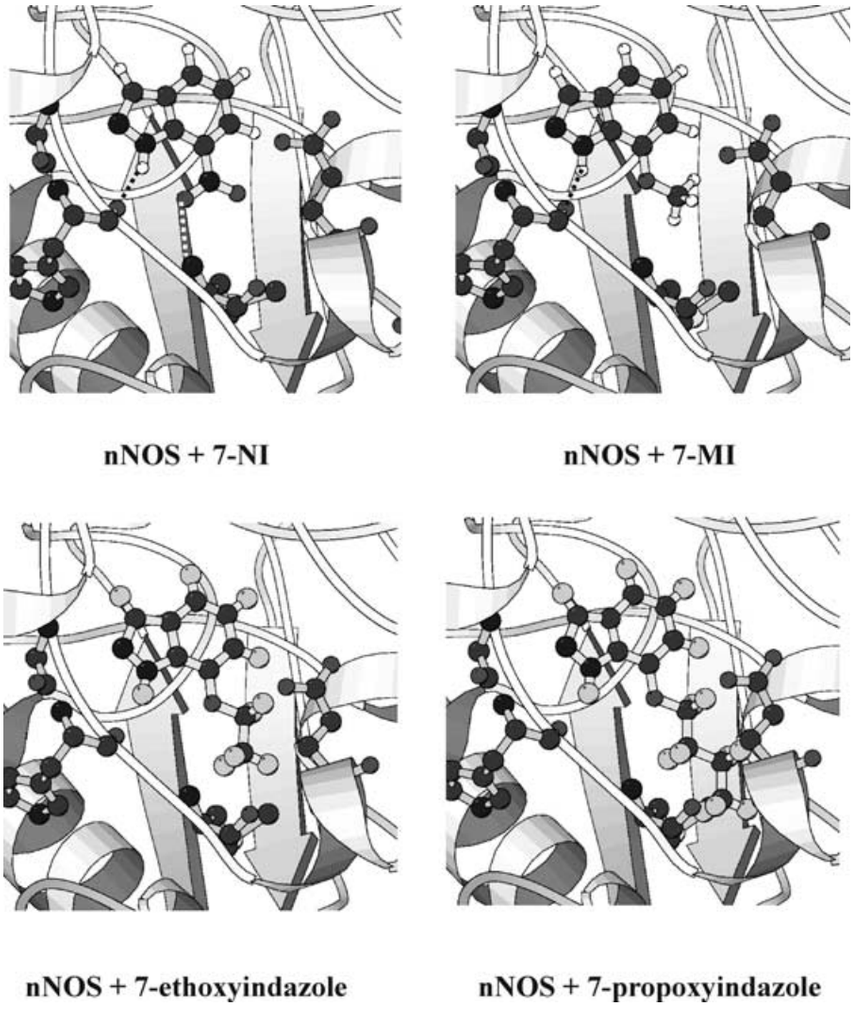


FIGURE 1 Detailed views of the binding cavity of nNOS:7-substituted indazole derivatives models. Dashed lines indicate hydrogen bonds. The figure was produced using Molscript.[19]

NOS is active whereas the monomer itself is inactive. The zinc ion plays a role in the dimerisation of NOSs.[6] The tetrahydrobiopterin cofactor (H4B) H-bonds with the same heme propionate, as does the substrate, which suggests the importance of a substrate-heme-cofactor synergy in the catalysed reaction.[17,18]

Most of NOS inhibitors, like the L-arginine analogues, or the thiourea compounds lie near the key glutamic acid of the substrate binding site. Contrarily, the imidazoles lie near both the glutamic acid and heme iron.[20] The structure of the catalytic heme domain of eNOS, complexed with an indazole inhibitor (3-Br-7-NI),[11] revealed a new mechanism of inhibition. At the substrate-binding site, 3-Br-7-NI does not bind directly either to the glutamic acid or to the heme iron. 3-Br-7-NI stacks parallel to the heme plane within van der Waals contact and its fixation in the binding cavity causes a displacement of the glutamic acid side chain from its original (potential substrate-binding position) at the edge of the cavity. Its position in the substrate-binding site is ensured by two H-bonds, one between the N–H of indazole and the carbonyl O of Trp 358, and a second between one oxygen atom from the nitro group and the peptide NH of Met 360.

The solved X-ray structure of 7-MI[20] showed that within the preferential conformation the methoxy group is oriented in the plane of the indazole ring outside of N–H. On the basis of the structure of the eNOS catalytic heme domain complexed with 3-Br-7-NI and the X-ray structure of 7-MI, a complex model of nNOS-7-MI was constructed. According to our model, 7-MI can interact in the same manner as 3-Br-7-NI. The 7-MI plane will stack with the heme plane in the same way as 3-Br-7-NI, since the 7-MI plane is situated within van der Waals contact distance from the heme. One H-bond between the oxygen of the methoxy group and the NH group of the methionine 360 is also conserved in the 7-MI-nNOS complex.

The constructed models in which the derivatives **3b–d** were positioned similarly to 3-Br-7-NI showed

that there is not sufficient space for the substituents on position-7 (see Figure 1).

This explanation does not apply to the derivative **3a**. Further investigations are in progress to understand the lack of effect of this derivative.

In conclusion, these studies suggest that indazole derivatives with a bulky substitutent on position-7 (larger than a methoxy group) will not be able to interact with nNOS in the same manner as 3-Br-7-NI and 7-MI. This result is in accordance with the experimentally measured biological activities of 7-ethoxyindazole, 7-propoxyindazole, 7-butoxyindazole, which are almost nonexistant (Table I).

Extension to the exploration of the effect of substitution on other positions (3, 4, 5 or 6) is still in progress.

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