Br. J. Pharmacol. (1993), **108**, 296–297

SPECIAL REPORT

7-Nitro indazole, an inhibitor of nitric oxide synthase, exhibits anti-nociceptive activity in the mouse without increasing blood pressure

[1]P.K. Moore, R.C. Babbedge, P. Wallace, Z.A. Gaffen & S.L. Hart

Pharmacology Group, Biomedical Sciences Division, King's College, University of London, Manresa Road, London SW3 6LX

7-Nitro indazole (7-NI) inhibits mouse cerebellar nitric oxide synthase (NOS) *in vitro* with an IC_{50} of 0.47 μM. Following i.p. administration in mice, 7-NI (10–50 mg kg^{-1}) produces dose-related anti-nociception as evidenced by an inhibition of late phase (15–30 min) but not early phase (0–5 min) hindpaw licking time following subplantar injection of formalin (10 μl, 5% v/v). The ED_{50} for this effect was 26 mg kg^{-1} (equivalent to 159.5 μmol kg^{-1}). Similar i.p. administration of 7-NI (20 and 80 mg kg^{-1}) in urethane-anaesthetized mice failed to increase MAP. Thus, 7-NI is a novel inhibitor of NOS which exhibits selectivity for the brain enzyme. Accordingly, 7-NI may be a useful starting point for the development of selective, centrally acting NOS inhibitors devoid of cardiovascular side effects and as a tool to study the central pharmacological effects of nitric oxide (NO).

Keywords: 7-Nitro indazole; L-N^{G}nitro arginine methylester (L-NAME); nitric oxide synthase; anti-nociception; formalin; blood pressure

Introduction L-N^G-nitro arginine methyl ester (L-NAME), a selective inhibitor of nitric oxide synthase (NOS), produces an opioid-independent anti-nociception in the mouse which is partially reversed by L-arginine (Moore *et al.*, 1991). That L-NAME is anti-nociceptive following i.c.v. administration in this species suggests a central mechanism of action. This conclusion is supported by electrophysiological studies in the rat which indicate a predominantly spinal site of action for L-NAME (Haley *et al.*, 1992).

L-NAME also inhibits vascular endothelial NOS resulting in a prolonged increase in blood pressure (e.g. Rees *et al.*, 1991). This action effectively precludes the use of L-NAME as an analgesic in man. In an attempt to identify selective inhibitors of brain NOS we have assessed the ability of a wide range of compounds to inhibit brain NOS *in vitro* and to increase mouse blood pressure. We describe here the results obtained using one such compound, 7-nitro indazole (7-NI).

Methods NOS activity was determined *in vitro* by the method of Dwyer *et al.* (1991). Mice (male, LACA, 28–32 g) were killed by cervical dislocation. Cerebella were removed, homogenized (1:10 v/v in 20 mM Tris buffer containing 2 mM EDTA, pH 7.4) and aliquots (25 μl) incubated (37°C) with L-arginine (120 nM) containing 0.5 μCi [^{3}H]-arginine (Amersham, sp. activity 66 Ci mmol^{-1}), NADPH (0.5 mM) and $CaCl_2$ (0.75 mM). Incubations also contained 7-NI (MIM, Research Chemicals Ltd.), L-NAME or L-N^G-monomethyl arginine (L-NMMA, Sigma) or an equal volume (5 μl) of 0.5% (w/v) sodium carbonate or distilled water as control. Final incubation volume was 105 μl. After 15 min, the reaction was stopped by addition of 3 ml HEPES buffer (20 mM containing 2 mM EDTA, pH 5.5) and the [^{3}H]-citrulline produced was separated by cation exchange chromatography on 0.5 ml columns of Dowex AG50-W8 Na^+ form (Sigma). [^{3}H]-citrulline was quantitated by liquid scintillation spectroscopy of duplicate 1 ml aliquots of the flow-through. In some experiments, mice were injected i.p. with 7-NI (25 mg kg^{-1}) or L-NAME (50 mg kg^{-1}) and killed 15 min thereafter. Cerebella were removed, homogenized and NOS activity determined as described above.

Anti-nociceptive activity of i.p. 7-NI was determined in mice by the formalin-induced hindpaw licking assay. Results show hindpaw licking time (s) in the early (0–5 min) and late phases (15–30 min) after subplantar injection of 10 μl formalin (5% v/v). In separate experiments, blood pressure of urethane (10 g kg^{-1})-anaesthetized mice was monitored for 45 min after i.p. administration of 7-NI. Full details of these methods have been published elsewhere (Moore *et al.*, 1991).

For *in vitro* experiments, 7-NI was dissolved in hot (80°C) sodium carbonate solution (0.5% w/v). 7-NI did not come out of solution on cooling. For *in vivo* experiments, 7-NI was suspended in arachis oil by sonication. Control animals received 10 ml kg^{-1} arachis oil or saline (0.9% NaCl, w/v). Results show mean ± s.e.mean. Statistical analysis was by Student's unpaired *t* test.

Results 7-NI potently inhibited mouse cerebellar NOS *in vitro* (IC_{50}, 0.47 ± 0.01 μM). For comparison, 7-NI was 1.8 times more potent than L-NAME (IC_{50}, 0.87 ± 0.02 μM) and 5 times more potent than L-NMMA (IC_{50}, 2.37 ± 0.03 μM) (Figure 1a). In separate experiments, administration of 7-NI (25 mg kg^{-1}, i.p.) decreased mouse cerebellar NOS activity measured 15 min thereafter by over 55% (3.9 ± 0.06 pmol citrulline mg^{-1} protein 15 min^{-1}, cf. 9.1 ± 0.26, arachis oil-injected controls, $n = 6$, $P < 0.01$). For comparison, a higher dose of L-NAME (50 mg kg^{-1}) produced only 46.2 ± 1.6% inhibition of this enzyme under identical conditions (4.46 ± 0.012 pmol citrulline mg^{-1} protein 15 min^{-1}, cf. 8.33 ± 0.15, saline-injected controls, $n = 6$, $P < 0.01$).

7-NI (10–50 mg kg^{-1}) also produced a dose-related inhibition of late phase formalin-induced hindpaw licking without influencing the early phase response (Figure 1b). The ED_{50} for 7-NI was 26.0 mg kg^{-1} (equivalent to 159.5 μmol kg^{-1}). In contrast, i.p. administration of 7-NI (25 and 80 mg kg^{-1}) did not increase MAP over the 45 min experimental period (e.g. 25 mg kg^{-1}, 47.4 ± 5.1 mmHg, cf. 51.6 ± 4.4 mmHg, $n = 4$, before 7-NI administration; 80 mg kg^{-1}, 43.9 ± 5.3 mmHg, cf. 49.5 ± 2.9 mmHg, $n = 4$, before 7-NI administration). In control experiments, i.p. administration of arachis oil failed to alter MAP.

[1] Author for correspondence.

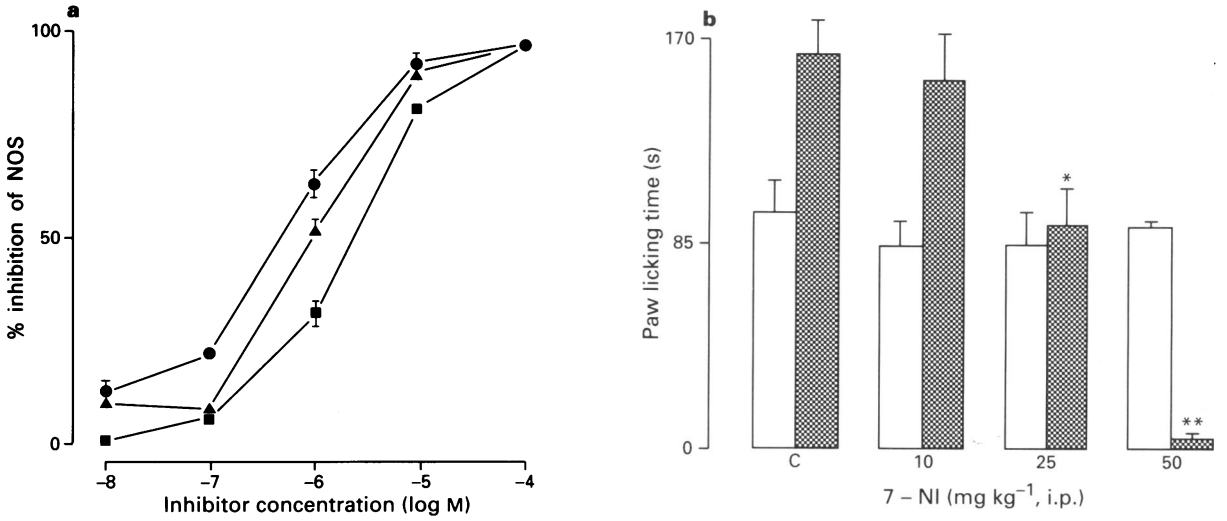

Figure 1 (a) Inhibition of mouse cerebellar nitric oxide synthase (NOS) by 7-nitro indazole (7-NI, ●), L-N^G-nitroarginine methyl ester (▲) and L-N^G-monomethyl arginine (■). Results show % inhibition of NOS and are mean ± s.e.mean, $n = 6$. Where no error bar is indicated error lies within dimensions of symbol. (b) Anti-nociceptive effect of 7-NI administered i.p. to mice 15 min before subplantar formalin injection. Open columns indicate early phase (0–15 min) whilst hatched columns indicate late phase (15–30 min) hindpaw licking times. Results show mean ± s.e.mean, $n = 6$–12, $^*P < 0.05$, $^{**}P < 0.01$. Control animals (labelled C) received 10 ml kg^{-1} arachis oil which, by itself, did not influence hindpaw licking time. (cf. saline-injected mice: early phase, 89.8 ± 7.0 s: late phase, 150.7 ± 11.5 s, $n = 15$).

Discussion We report here that 7-NI inhibits mouse cerebellar NOS. To the best of our knowledge 7-NI is the first potent inhibitor of this enzyme which is not a guanidino-substituted derivative of L-arginine. The chemical characteristics of 7-NI which confer NOS inhibitory activity remain to be determined although the presence of two adjacent nitrogen atoms in the pyrazole ring does bear some similarity to the arrangement of the guanidino terminus of L-arginine. However, pyrazole itself is inactive (Babbedge & Moore, unpublished) suggesting that the fused ring structure of the indazole nucleus is necessary for activity. We therefore propose that 7-NI may be useful as a tool to study the pharmacological actions of NO.

The finding that 7-NI, like L-NAME (Moore *et al.*, 1991), causes anti-nociception in the mouse provides further support for our hypothesis that NO plays a role in pain appreciation. In this context, it is of interest that 7-NI and L-NAME exhibit (a) similar anti-nociceptive potency in the mouse (ED_{50} for L-NAME, 186 μmol kg^{-1}; see Morgan *et al.*, 1992) and (b) similar ability to inhibit mouse cerebellar NOS. Interestingly, administration of 7-NI (25 mg kg^{-1}) to intact mice results in a greater inhibition of cerebellar NOS than does similar injection of a higher (50 mg kg^{-1}) dose of L-NAME. Further research to investigate the relative pharmacokinetic profiles of 7-NI and L-NAME following i.p. injection in mice are required.

Unlike, L-NAME, 7-NI does not cause an increase in MAP indicating a lack of effect on endothelial cell NOS activity in this species. These results show that 7-NI exhibits selectivity for the brain NOS enzyme thereby highlighting at least one difference between the brain and endothelial cell isoforms of this enzyme. It will clearly be of interest to determine the effect of 7-NI on other constitutive and inducible isoforms of NOS. The lack of cardiovascular side effects of 7-NI reported in this study, if confirmed in other species, is potentially of clinical interest. For example, the development of a selective inhibitor of brain NOS may be of therapeutic use not only for analgesia but for other central nervous system disorders in which over-production of NO is believed to play a causative role e.g. neurodegenerative disease (see Garthwaite, 1991).

We thank the MRC for financial support. R.C.B. is an SERC-CASE scholar with Wellcome Research Laboratories.

References

DWYER, M.A., BREDT, D.S. & SNYDER, S.L. (1991). Nitric oxide synthase: irreversible inhibition by L-N^G-nitro arginine in brain in vitro and in vivo. *Biochem. Biophys. Res. Commun.*, **176**, 1136–1141.

GARTHWAITE, J. (1991). Glutamate, nitric oxide and cell-cell signalling in the nervouse system. *Trends Neurosci.*, **14**, 60–67.

HALEY, J.E., DICKENSON, A.H. & SCHACTER, M. (1992). Electrophysiological evidence for a role of nitric oxide in prolonged chemical nociception in the rat. *Neuropharmacology*, **31**, 251–258.

MOORE, P.K., OLUYOMI, A.O., BABBEDGE, R.C., WALLACE, P. & HART, S.L. (1991). L-N^G-nitro arginine methyl ester exhibits anti-nociceptive activity in the mouse. *Br. J. Pharmacol.*, **102**, 198–202.

MORGAN, C.V.J., BABBEDGE, R.C., GAFFEN, Z.A., WALLACE, P., HART, S.L. & MOORE, P.K. (1992). Synergistic anti-nociceptive effect of L-N^G-nitro arginine methyl ester (L-NAME) and flurbiprofen in the mouse. *Br. J. Pharmacol.*, **106**, 493–497.

REES, D.D., PALMER, R.M.J. & MONCADA, S. (1991). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3375–3378.

(Received October 19, 1992
Accepted November 16, 1992)