British Journal of Pharmacology (1995) 116, 2349-2350

SPECIAL REPORT

The antinociceptive effect of 1-(2-trifluoromethylphenyl) imidazole (TRIM), a potent inhibitor of neuronal nitric oxide synthase *in vitro*, in the mouse

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1-(2-trifluoromethylphenyl)imidazole (TRIM) is a potent inhibitor of neuronal (mouse cerebellar) and inducible (lung from endotoxin-pretreated rats) isoforms of nitric oxide synthase (NOS) with IC_{50} values of 28.2 μM and 27.0 μM, respectively. In contrast, TRIM is a poor inhibitor of bovine aortic endothelial NOS with an IC_{50} of 1057.5 μM. TRIM (10-50 mg kg^{-1}) administered i.p. exhibits dose-related antinociceptive activity in the mouse (assessed as inhibition of late phase formalin-induced hindpaw licking behaviour) with an ED_{50} of 85.8 μmol kg^{-1}. In contrast, TRIM (50 mg kg^{-1}, i.p.) failed to influence mean arterial blood pressure in the urethane-anaesthetized mouse. Thus, TRIM may be of use as an experimental tool with which to investigate the biological roles of nitric oxide (NO) within the central nervous system.

Keywords: 1-(2-trifluoromethylphenyl)imidazole; nitric oxide; nitric oxide synthase; antinociception

Introduction Nitric oxide synthase (NOS), which catalyses the conversion of L-arginine into NO and citrulline, exists as a number of structurally distinct isoforms i.e. neuronal (nNOS), endothelial (eNOS) and inducible (iNOS), thereby raising the possibility that isoform-selective inhibitors of this enzyme may prove useful for the treatment of a wide variety of disease states (Ogden & Moore, 1995). In this context, we have previously shown that 7-nitro indazole (7-NI) is an inhibitor of nNOS *in vitro* with potent antinociceptive activity *in vivo* but is devoid of vasopressor activity in the anaesthetized mouse (Moore *et al.*, 1993). Despite the lack of cardiovascular side effects of this compound *in vivo* 7-NI is also a potent inhibitor of bovine aortic endothelial eNOS *in vitro* (Babbedge *et al.*, 1993). In an attempt to identify additional NOS inhibitors with more marked *in vitro* selectively for nNOS (c.f. eNOS) we have examined a number of nitrogen-containing heterocyclic compounds for ability to inhibit NOS *in vitro*. We now demonstrate that one such compound, 1-(2-trifluoromethylphenyl)imidazole (TRIM), is a potent and relatively selective inhibitor of nNOS both *in vitro* and *in vivo*.

Methods The methods used in this study have been described elsewhere (Babbedge *et al.*, 1993; Moore *et al.*, 1993). NOS activity was determined *in vitro* in homogenates (10,000 *g*) of mouse (male, LACA, 25-30 g) cerebella, lungs from urethane-anaesthetized (10 g kg^{-1}, i.p.) rats (male, Wistar, 280-350 g) 6 h after i.p. administration of 5 mg kg^{-1} *E. Coli* endotoxin (serotype: 0127-B8) and bovine aortic endothelial cells as the source of nNOS, iNOS and eNOS isoforms respectively. Homogenate (25 μl) was incubated (37°C, 15 min) with L-arginine (120 nM) containing 0.5 μCi [^{3}H]-L-arginine (Amersham, sp. act. 68 Ci mmol^{-1}), NADPH (0.5 mM) and either TRIM (0.1-1250 μM, MTM Lancaster Ltd.), L-N^G nitro arginine methyl ester (L-NAME, 0.1-100 μM, Sigma) or an equal volume (5 μl) of distilled water. Incubations of nNOS and eNOS (but not iNOS) enzyme also contained $CaCl_2$ (0.75 mM). The reaction was stopped with 3 ml HEPES buffer (20 mM containing 2 mM EDTA, pH 5.5) and the [^{3}H]-citrulline formed separated from unreacted [^{3}H]-L-arginine on 0.5 ml columns of Dowex AG50-W8 Na^+ form (Sigma). [^{3}H]-citrulline was quantitated by liquid scintillation spectroscopy.

Antinociceptive activity of i.p. administered TRIM was assessed as the ability to inhibit formalin-induced hindpaw licking behaviour in the mouse. 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). TRIM or saline (0.9% NaCl w/v) was administered 15 min before the formalin injection. In experiments to assess the reversibility of the antinociceptive effect of TRIM animals received either L-arginine (50 mg kg^{-1}, Sigma) or saline (both i.p.) 5 min before the TRIM injection. In separate experiments, blood pressure of urethane (10 g kg^{-1}, i.p.)-anaesthetized mice was monitored continuously for 45 min after i.p. administration of TRIM (50 mg kg^{-1}). Results are shown as mean ± s.e.mean. Statistical analysis was by ANOVA with *post-hoc* Tukey test for multiple comparisons.

Results TRIM inhibited mouse cerebellar nNOS and rat lung iNOS *in vitro* with IC_{50} values of 28.2 ± 0.5 μM and 27.0 ± 0.9 μM (both $n=6$), respectively (Figure 1a). For comparison, TRIM was 43 times and 2.5 times less potent than L-NAME as an inhibitor of nNOS (IC_{50}, 0.66 ± 0.06 μM, $n=6$) and iNOS (IC_{50}, 10.6 ± 0.4 μM, $n=6$). In contrast, TRIM was a very weak inhibitor of bovine aortic endothelial eNOS (IC_{50}, 1057.5 ± 12.2 μM, $n=6$ c.f. L-NAME, IC_{50}, 6.5 ± 0.06 μM, $n=6$) (Figure 1a).

TRIM (10-50 mg kg^{-1}, i.p.) also caused dose-related inhibition of late phase formalin-induced hindpaw licking (Figure 1b). Only at the highest dose of TRIM was an inhibition of the early phase response observed. The ED_{50} for inhibition of late phase hindpaw licking was 85.8 μmol kg^{-1}. In control experiments, L-arginine (50 mg kg^{-1}, i.p.) failed to influence either phase of hindpaw licking (Figure 1b) but reversed the late phase antinociceptive effect of TRIM (20 mg kg^{-1}, i.p.). No discernible changes in animal behaviour (e.g. sedation, motor ataxia, locomotor or exploratory/grooming activity) was apparent in animals administered TRIM.

The resting mean arterial blood pressure (MAP) of urethane-anaesthetized mice was 52.3 ± 3.3 mmHg ($n=9$). Administration of TRIM (50 mg kg^{-1}, i.p.) failed to influence MAP over 45 min (MAP, 46.7 ± 6.1 mmHg, $n=4$, determined 45 min after TRIM injection c.f. 42.1 ± 2.7 mmHg, $n=5$, at the same time after saline injection).

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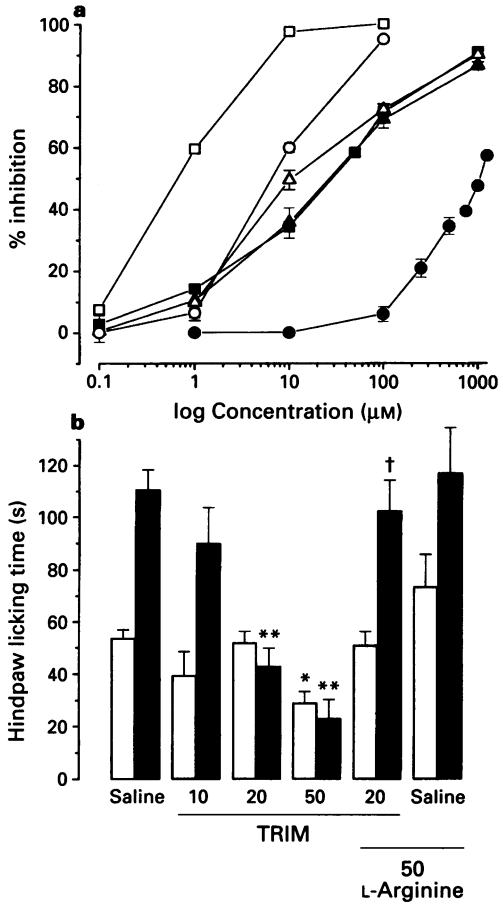

Figure 1 (a) Effect of 1-(2-trifluoromethylphenyl)imidazole (TRIM) (closed symbols) and L-N^G-nitro arginine methyl ester (L-NAME) (open symbols) on mouse cerebellar nNOS (■, □), rat lung iNOS (▲, △) and bovine aortic endothelial eNOS (●, ○). Results show % inhibition of [^{3}H]-citrulline formation and are mean ± s.e.mean, $n=6$. The absence of error bars indicates errors lie within dimensions of symbols. (b) Antinociceptive effect of TRIM (mg kg^{-1}, i.p.) determined as effect on early phase (open columns) and late phase (solid columns) formalin-induced hindpaw licking behaviour in mice. L-Arginine (50 mg kg^{-1}, final pair of columns) did not influence formalin-induced hindpaw licking behaviour but reverses the antinociceptive effect of TRIM (20 mg kg^{-1}). Results indicate hindpaw licking time in s and are mean ± s.e.mean, $n=5-14$, $^*P<0.05$, $^{**}P<0.01$ c.f. saline-injected animals, †$P<0.05$ c.f. TRIM (20 mg kg^{-1})-injected animals.

Discussion To the best of our knowledge TRIM is the first reported imidazole-based NOS inhibitor to exhibit substantial isoform selectivity. Compared to eNOS, TRIM is a relatively selective inhibitor of nNOS and iNOS with potency ratios *in vitro* of 37 and 39 respectively. Such a profile of NOS isoform selectivity *in vitro* presumably underlies the ability of TRIM to inhibit formalin-induced hindpaw licking *in vivo* in the absence of vasopressor activity in the mouse. The antinociceptive effect of TRIM observed in this study most probably results from inhibition of nNOS in neurones of the dorsal horn of the spinal cord (see Moore *et al.*, 1993). However, an effect on iNOS cannot be excluded. Since TRIM is a less potent inhibitor of nNOS *in vitro* than either L-NAME (this study) or 7-NI (Moore *et al.*, 1993) it is perhaps surprising that TRIM is twice as potent as these compounds with respect to antinociceptive activity (Moore *et al.*, 1993). The reason for this discrepancy between *in vitro* and *in vivo* data is unknown. However, it is conceivable that higher concentrations of TRIM (c.f. L-NAME or 7-NI) are achieved in the central nervous system following i.p. administration. The precise site of interaction of TRIM with NOS also remains to be determined although an effect on the haem and/or calmodulin binding sites of this enzyme seems likely considering the proposed NOS binding site of imidazole (Wolff *et al.*, 1993).

There have been few accounts of NOS inhibitors with selectivity for the nNOS isoform (c.f. eNOS). S-methyl L-thiocitrulline, which is reportedly 10 times more selective for nNOS *in vitro* (Furfine *et al.*, 1994), has recently been shown to inhibit endothelium-dependent vasorelaxation in isolated aortic rings indicative of significant eNOS inhibitory activity (Joly *et al.*, 1995). Furthermore, Garvey and colleagues (1994) described several bisisothiourea compounds with 30–70 fold selectivity for nNOS (c.f. eNOS) *in vitro* although the apparent toxicity and inability of these compounds to cross cell membranes may preclude their use *in vivo*.

The present results raise the possibility that TRIM may be a useful tool with which to investigate the biological significance of NO synthesized by nNOS (and perhaps iNOS). The possibility that TRIM may have therapeutic potential particularly in those central nervous system disorders which have been associated with excessive NO biosynthesis (e.g. algesia, neurodegeneration) should also be considered.

We thank the Arthritis and Rheumatism Research Council and the Wellcome Trust for financial support.

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(Received July 5, 1995
Accepted August 17, 1995)