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Isothioureas: potent inhibitors of nitric oxide synthases with variable isoform selectivity

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1 The induction of a calcium-independent isoform of nitric oxide (NO) synthase (iNOS) and a subsequent enhanced formation of NO has been implicated in the pathophysiology of a variety of diseases including inflammation and circulatory shock. Here we demonstrate that the S-substituted isothioureas, S-methylisothiourea (SMT), S-(2-aminoethyl)isothiourea (aminoethyl-TU), S-ethylisothiourea (ethyl-TU) and S-isopropylisothiourea (isopropyl-TU) potently inhibit iNOS activity in J774.2 macrophages activated with bacterial endotoxin with EC_{50} values 8–24 times lower than that of N^G-methyl-L-arginine (MeArg) and 200-times lower than that of N^G-nitro-L-arginine (L-NO_2Arg).

2 The inhibition of iNOS activity by these S-substituted isothioureas is dose-dependently prevented by excess of L-arginine suggesting that these isothioureas are competitive inhibitors of iNOS at the L-arginine binding site.

3 Ethyl-TU and isopropyl-TU are 4–6 times more potent than MeArg in inhibiting the constitutive NOS activity in homogenates of bovine aortic endothelial cells (eNOS) and are more potent pressor agents than MeArg in the anaesthetized rat. SMT is equipotent with MeArg, whereas aminoethyl-TU is 6-times less potent in inhibiting eNOS activity *in vitro*. Both SMT and aminoethyl-TU, however, elicit only weak pressor responses (approximately 15 mmHg at 10 mg kg^{-1}, i.v.) *in vivo*.

4 A comparison of the potencies of ethyl-, iso-propyl-, n-propyl-, t-butyl- and n-butyl-isothioureas on iNOS activity shows that the inhibitory activity of S-substituted isothioureas declines sharply if the side chain exceeds 2 carbon atoms in length. Similarly, substitution of the ethylene side chain of ethyl-TU also results in a diminished potency. Substitution of either one or both nitrogens of SMT with either amino or alkyl groups also substantially reduces its NOS inhibitory potency.

5 In conclusion, isothioureas represent a new class of NOS inhibitors which includes the most potent inhibitors of iNOS activity reported to date. Some members of this class (ethyl-TU and isopropyl-TU) are potent inhibitors of eNOS and iNOS with little selectivity towards either isoform, while others (SMT and aminoethyl-TU) are relatively selective inhibitors of iNOS activity. These latter agents may become useful tools for studying the role of iNOS in various disease models and may be useful in the therapy of diseases that are associated with an enhanced formation of NO due to iNOS induction, such as inflammation, circulatory shock or cancer.

Keywords: Nitric oxide; constitutive nitric oxide synthase; inducible nitric oxide synthase; blood pressure; circulatory shock; inflammation; endotoxin

Introduction

The induction of a distinct isoform of nitric oxide synthase (inducible NOS, iNOS) in various cells by agents such as bacterial lipopolysaccharide (LPS), interleukin-1, tumour necrosis factor and γ-interferon (IFN), leads to an enhanced formation of nitric oxide (NO) from its precursor, L-arginine. The NO produced may have physiological roles, for instance in the antimicrobial activity of activated macrophages (Moncada *et al.*, 1991; Nathan, 1992), but the sustained production of large quantities of NO following the induction of iNOS has been implicated in the pathogenesis of circulatory shock (Thiemermann & Vane, 1990; Meyer *et al.*, 1992; Nava *et al.*, 1992; Szabó *et al.*, 1993a; Thiemermann *et al.*, 1993; Szabó & Thiemermann, 1994; Thiemermann, 1994), diabetes (Corbett *et al.*, 1992), chronic inflammation (McCartney-Francis *et al.*, 1993; Stefanovic-Racic *et al.*, 1993; Miller *et al.*, 1993; Koprowski *et al.*, 1993; Vane *et al.*, 1994), transplant rejection (Langrehr *et al.*, 1993) and cancer (Piper *et al.*, 1993). Inhibition of iNOS activity may, therefore, be of significant therapeutic benefit in these conditions. However, the L-arginine based inhibitors of NOS currently used in experimental models and proposed for therapeutic intervention also inhibit the constitutive isoforms of NOS present in the brain (nNOS) or in endothelial cells (eNOS).

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NO from endothelial NOS (eNOS) is involved in the regulation of blood pressure and organ blood flow distribution and inhibits the adhesion of platelets and polymorphonuclear granulocytes to the endothelial surface (Moncada *et al.*, 1991; Nathan, 1992; Dinerman *et al.*, 1993; Calver *et al.*, 1993; Vane, 1994; Lowenstein *et al.*, 1994). Dysfunction of NO formation by the vascular endothelium has been shown in a number of vascular and non-vascular diseases (Fujiwara *et al.*, 1986; Bucala *et al.*, 1991; Szabó *et al.*, 1992; Parker & Adams, 1993). In view of the physiological roles of eNOS and the pathophysiological implications of enhanced formation of NO by iNOS, pharmacological agents that selectively inhibit iNOS may well have a therapeutic advantage over those currently available. The L-arginine analogues N^G-cyclopropyl-L-arginine, N^G-methyl-L-arginine (MeArg), N^G-nitro-L-arginine (L-NOArg) and its methyl ester (after hydrolysis) are more potent inhibitors of eNOS than of iNOS, while MeArg, N^G-aminohomo-L-arginine and N^G-amino-L-arginine are equipotent inhibitors of both eNOS and iNOS (Gross *et al.*, 1990; 1991; Lambert *et al.*, 1991; 1992). Aminoguanidine and some related guanidines, however, are weaker inhibitors of eNOS than iNOS, and, hence, exhibit selectivity as inhibitors of iNOS (Corbett *et al.*, 1992; Misko *et al.*, 1993; Hasan *et al.*, 1993). Here we describe a novel class of potent NOS inhibitors, some of which show relative selectivity towards iNOS.

Methods

Cell culture

The mouse macrophage cell line J774.2 was cultured in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine (4×10^{-3} M) and 10% foetal calf serum (FCS) as described (Szabó *et al.*, 1993b; 1994b). Cells were cultured in 96-well plates with culture medium (200 µl/well) until they reached confluence. To induce iNOS, fresh culture medium containing *E.coli* lipopolysaccharide (LPS, 1 µg ml^{-1}) was added. Nitrite accumulation in the cell culture medium was measured after 24 h. To assess the effects of various agents on the production of nitrite, they were added to the medium 6 h after induction of iNOS with LPS. At this time there is no detectable increase in the concentration of nitrite, and agents such as glucocorticoids, that inhibit the induction but not the activity of iNOS, have no effect on subsequent nitrite production (Szabó *et al.*, 1993b, 1994b). In some experiments, agents were applied together with excess L-arginine (in total 1.4, 10 or 30 mM in the culture medium, as opposed to 0.4 mM in normal DMEM).

Nitrite measurement

Nitrite production, an indicator of NO synthesis, was measured in the supernatant of J774.2 macrophages as previously described (Szabó *et al.*, 1993b; 1994b). Nitrite was measured by adding 100 µl of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) to 100 µl samples of cell culture medium. The optical density at 550 nm (OD_{550}) was measured with an Anthos 1.2.1 microplate reader (Anthos Labtechnik, Salzburg, Austria). Nitrite concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite (5–100 µM) prepared in culture medium.

Cell viability and respiration

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Szabó *et al.*, 1993b, 1994b). Cells in 96-well plates were incubated (37°C) with MTT (0.2 mg ml^{-1}) for 60 min. Culture medium was removed and the cells solubilized in DMSO (100 µl). The extent of reduction of MTT to formazan within cells was quantitated by measurement of OD_{550} with the Anthos microplate reader. Formazan production was expressed as a percentage of the values obtained from untreated cells. Inhibition of nitrite production by the drugs tested is only reported where there is no significant decrease in cell viability.

The hydrolysis of substituted isothioureas to thiols and urea

The rate of hydrolysis of S-substituted isothioureas at the pH of the experiments was estimated by measuring the release of free thiols (R_1-SH, see Figure 1). Isothioureas (0.5–5 mM) were incubated in 96-well plates at pH 7.4 or 12 in the presence of 2,2'-dithiodipyridine (Aldrithiol-2, 1 mM, at 37°C). Reduction of Aldrithiol-1 by the released thiol was measured by increased absorbance at 340 nm using the Anthos microplate reader. In addition, urea from the hydrolysis of S-methylisothiourea (SMT) (incubated at 37°C for 17 h at pH 7.4 or 12) was determined by heating for 10 min at 100°C with 2,3-butanedione monoxime under strongly acidic conditions and measuring spectrophotometrically at 405 nm.

Nitric oxide synthase assay

The intimal surfaces of fresh bovine aortae were scraped in the presence of buffer composed of (mM): Tris·HCl 50, EDTA 0.1, EGTA 0.1, 2-mercaptoethanol 12 and phenylmethylsulphonyl fluoride 1 (pH 7.4). Cell suspensions were then homogenized in the buffer (composition as above) on ice in an Ultra-Turrax T25 homogenizer (Janke & Kunkel, IKA Labortechnik, Staufen i. Br., Germany). Conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in the homogenates as described (Szabó *et al.*, 1993a; Thiemermann *et al.*, 1993). Briefly, cell homogenate (50 µl) was incubated in the presence of [^{3}H]-L-arginine (10 µM, 5 kBq/tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 µM) and calcium (2 mM) for 20 min at 37°C in HEPES (20 mM) buffer (pH 7.5). Reactions were stopped by dilution with 1 ml of ice cold HEPES (20 mM) buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the eluted [^{3}H]-L-citrulline activity was measured by scintillation counting (Beckman, LS3801; Fullerton, CA, U.S.A.).

Haemodynamic measurements

Male Wistar rats (260–320 g; Glaxo Laboratories Ltd., Greenford, Middx.) were anaesthetized with thiopentone sodium (Trapanal, 3% solution; 120 mg kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket (BioSciences, Sheerness, Kent, U.K.). The right carotid artery was cannulated and connected to a pressure transducer (P23XL, Spectramed, Stratham, U.S.A.) for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate which were displayed on a Grass model 7D polygraph recorder (Grass Instruments, Quincy, Mass, U.S.A.) (Thiemermann & Vane, 1990; Szabó *et al.*, 1993a). Left and right femoral veins were cannulated for the administration of drugs. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 10 min. After recording baseline haemodynamic parameters, animals received NOS inhibitors in a cumulative manner (0.001–10 mg kg^{-1}, i.v. bolus injections) with 5 min between injections.

Materials

Aminoguanidine, bacterial lipopolysaccharide (*E. coli*, serotype No. 0127:B8), calmodulin, DMEM, L-glutamine, MTT, NADPH, N^G-nitro-L-arginine, N^G-nitro-L-arginine methyl ester, sodium nitrite and Dowex 50W anion exchange resin were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). N^G-methyl-L-arginine monoacetate (MeArg) was obtained from Calbiochem (Nottingham, U.K.). Tetrahydrobiopterin was from Dr B. Schirks Laboratories, Jona, Switzerland. Foetal calf serum was obtained from Advanced Protein Products (West Midlands, U.K.). L-[2,3,4,5-^{3}H]-arginine hydrochloride was obtained from Amersham (Buckinghamshire, U.K.). N^G-amino-L-arginine was synthesized by Dr O.W. Griffith (Medical College of Wisconsin, Milwaukee U.S.A.). Methanethiol sodium salt, methyl-methanethiolsulphonate and methane disulphide were obtained Fluka (Gillingham, Dorset, U.K.). 2,3 Butanedione monoxime and 2,2'-dithiodipyridine (Aldrithiol), were obtained from Aldrich (Gillingham, Dorset, U.K.).

Drugs for both *in vitro* and *in vivo* experiments were dissolved in sterile, saline. Picrate salts (see Table 1) for use with cultured macrophages were first dissolved in dimethylsulphoxide (Sigma) and then diluted with 9 parts water. Neither the mode of dissolution nor the type of salt used (bromide, picrate etc.) had any influence on the inhibitory activity of a particular isothiourea.

Isothioureas

Compounds No. **6–8**, **19**, **25**, **29**, **30–34**, **43** and **44** in Table 1 were purchased from Aldrich. The other S-substituted isothioureas were generally prepared as salts from the appro-

priate thioureas or thiosemicarbazides (Aldrich) by reaction with alkyl or substituted alkyl halides by variations on established methods. In some cases, alkylation was achieved using alkyl *p*-toluenesulphonates. Typically, thiourea or the appropriate N-substituted thiourea was dissolved in boiling alcohol (isopropanol or ethanol) and refluxed for several hours with a slight excess of the appropriate alkyl halide or alkyl *p*-toluenesulphonate. Solutions were allowed to cool. Solvent and volumes were chosen to encourage crystallization of the product at this stage. Otherwise, the solvent was removed and the residues worked up into solid and recrystallized. For those compounds that did not crystallize as the hydro-halide salts, their picrate salts were precipitated from aqueous solution. For detailed synthetic procedures the reader is referred to the following reviews (and references therein); Schroeder, 1955; Reid, 1963 and to Olin & Dains, 1930. Elemental

Table 1 Inhibitory effect of substituted isothioureas and reference compounds on nitrite production by activated macrophages

	Reference compound		$^{a}EC_{50}$ (μM)
1	N^{G}-methyl-L-arginine (MeArg)		48
2	N^{G}-nitro-L-arginine (L-NOArg)		400
3	N^{G}-nitro-L-arginine methyl ester (L-NAME)		>1000 (^{b}32%)
4	N^{G}-amino-L-arginine		90
5	Aminoguanidine		130
	Compound		
	$*R_2 = R_3 = R_4 = H$)	$*R_1$	EC_{50} (μM)
6	Thiourea	–	>1000 (^{b}26%)
7	SMT	CH_3-	6
8	Ethyl-TU	CH_3-CH_2-	2
9	iso-Propyl-TU	CH_3-(CH_3-)CH_2-	2
10	n-Propyl-TU	CH_3-$(CH_2)_2$-	78
11	t-butyl-TU	$(CH_3)_3C$-	80
12	n-Butyl-TU	CH_3-$(CH_2)_3$-	280
13	sec-butyl-TU	CH_3-CH_2-(CH_3-)CH-	65
14	n-Pentyl-TU	CH_3-$(CH_2)_4$-	>1000 (^{b}46%)
15	Cyclopentyl-TU	cyclopentyl-	242
16	Isoamyl-TU	CH_3-(CH_3-)$(CH_2)_3$-	900
17	Lauryl-TU	CH_3-$(CH_2)_{11}$-	>10^{c}
18	Cetyl-TU	CH_3-$(CH_2)_{15}$-	>1000 (^{b}29%)
19	Benzyl-TU	$C_6H_5CH_2$-	100
20	4-Chlorobenzyl-TU	4-Cl-$C_6H_4CH_2$-	65
21	Chloroethyl-TU	Cl-CH_2-CH_2-	28
22	Cyanomethyl-TU	NC-CH_2-	>1000
23	Acetimido-TU	NH_2-CO-CH_2-	>1000 (^{b}6%)
24	Carboxy-methyl-TU	CO_2H-CH_2-	>1000 (^{b}21%)
25	Aminoethyl-TU	NH_2-CH_2-CH_2-	3
26	Methoxy-ethyl-TU	CH_3-O-CH_2-CH_2-	68
27	Ethoxy-ethyl-TU	CH_3-CH_2-O-CH_2-CH_2-	170
28	Phenoxy-propyl-TU	C_6H_5-O-CH_2-CH_2-CH_2-	200
29	Amidine-thiopropane-sulphonic acid	SO_3H-CH_2-CH_2-CH_2-	>1000 (^{b}29%)
30	3-Indolyl-TU	Indolyl	>10^{c}
	$R_2 = R_3 = H$	R_1 R_4	EC_{50} (μM)
31	2-Aminothiazoline	-CH_2-CH_2-^{d}	38
32	2-Aminothiazole	-CH_2 = CH_2-	470
33	2-Amino-1,3,4-thiadiazol	-CH_2-NH-	>1000
34	Pseudothiohydantoin	-CH_2-CO-	>1000
	R_1 = CH3, R_3 = H	R_2 R_4	EC_{50} (μM)
35	N-methyl-SMT	CH_3-, H	350
36	N,N'-dimethyl-SMT	CH_3-, CH_3	>1000 (^{b}43%)
37	N,N'-diethyl-SMT	CH_3-CH_2-, CH_3-CH_2-	>1000 (^{b}29%)
38	N-phenyl-SMT	C_6H_5-, H-	330
39	N-(4-nitrophenyl)SMT	4-NO_2-C_6H_4-, H-	310
40	N-amino-SMT	NH_2-, H-	630
41	N-amino-N'-methyl-SMT	NH_2- CH_3-	220
42	N-amino-N'-phenyl-SMT	NH_2- C_6H_5-	550
43	2-(Methylthio)imidazoline	-CH_2-CH_2-^{e}	860
44	N-cyano-N'-methyl-SMT	NC- CH_3-	>1000
	R_1 = CH3; R_4 = H	R_2 R_3	EC_{50} (μM)
45	N-amino-N-methyl-SMT	NH_2- CH_3	150

$*R_{1-4}$ refer to Figure 1. $^{a}EC_{50}$s calculated from 5 point dose-response curves (100 nM–1 mM). ^{b}Inhibition did not exceed 50% at 1 mM; percentage inhibition is given at 1 mM. ^{c}Lauryl-TU and 3-indolyl-TU are cytotoxic over 10 μM. ^{d}Cyclic compound: R_1 (^{e} or R_2) and R_4 are replaced by chain indicated to give a 5-membered ring. The compounds were used as the following salts: acetate (.CH_3CO_2H): **1**, **4**; chloride (.HCl): **5**, **19**, **20**; bromide (.HBr): **8**, **9**, **14–18**, **21–25**, **27**, **28**; iodide (.HI): **12**, **30**, **35**, **36**, **38**; *p*-toluene sulphonate (.HSO_3-C_6H_4-CH_3): **37**, **41**, **42**, **45**; sulphate (.1/2 H_2SO_4): 7; and picrate: **10**, **11**, **13**, **26**.

(C H N) compositions were all within 5% of theoretical. Melting points were found to be the same as available literature values.

Note that the nomenclature does not necessarily follow IUPAC conventions: names are intended to emphasize the group attached to the sulphur atom (R_1, Figure 1). N-substituted compounds are treated as derivatives of the parent compound, SMT, and are prefixed by N-. S-substituted-isothioureas may also be described as 2-substituted-thiopseudoureas.

Statistical evaluation

All values in the figures and text are expressed as mean ± s.e.mean of n observations. For each drug, at least 9 wells from 3 independent experiments were studied. Student's unpaired t test was used to compare means between groups. A P-value less than 0.05 was considered to be statistically significant.

Results

Many of the S-substituted isothioureas tested cause a dose-dependent inhibition of nitrite production by stimulated J744.2 macrophages, with a wide range of potencies (Table 1). They appear to do so by inhibiting the activity of iNOS, rather than its induction, for they inhibit nitrite accumulation when added to the medium at a time point when induction of iNOS has already occurred (i.e. 6 h after addition of LPS). The most potent isothioureas are those with only short alkyl chains on the sulphur atom ($R_1 = C_1$ or C_2, where R refers to Figure 1) and no substituents on the nitrogens ($R_2 = R_3 = R_4 = H$). Some of these have potencies of up to 24 times that of MeArg. A comparison of dose-response curves (Figure 2) shows the following order of potency for these compounds as inhibitors of iNOS (emboldened numbers refer to Table 1): S-ethyl-isothiourea (ethyl-TU, **8**) = S-isopropyl-isothiourea (isopropyl-TU, **9**) > S-(2-aminoethyl)isothiourea (aminoethyl-TU, **25**) > SMT (**7**) >> N^G-methyl-L-arginine (MeArg,**1**) >> N^G-nitro-L-arginine (L-NOArg, **2**). Increasing the length of R_1 (see Figure 1) or incorporating polar groups into R_1 results in diminished potencies of the isothioureas, with the notable exception of aminoethyl-TU (**25**). However, several compounds, e.g. chloroethyl-TU (**21**), methoxyethyl-TU (**26**) and 2-aminothiazoline (**31**) are of similar potency to MeArg (**1**) as inhibitors of iNOS activity (Table 1).

Increasing concentrations of L-arginine (1–30 mM) in the medium of macrophages treated with isothioureas caused a dose-dependent increase in the EC_{50} values of MeArg and selected isothiourea derivatives (Table 2).

To investigate the possible liberation of thiols (R_1-SH) deriving from the hydrolysis of isothioureas and their potential effects on iNOS activity, compounds that release methanethiol (a product of the hydrolysis of SMT) were tested for their inhibition of nitrite production in activated macrophages. At 0.1 mM, sodium methanethiolate and methyl-methanethiolsulphonate inhibited nitrite accumulation by 35 ± 3% and 45 ± 2%, respectively. At 1 mM, sodium methanethiolate and methane disulphide caused a 41 ± 9% and 32 ± 2% inhibition, respectively, and methyl-methanethiolsulphonate became cytotoxic ($n = 4–6$). Thus, these agents appear to be approximately 100 fold weaker than SMT as inhibitors of iNOS activity, and, hence, release of methanethiol is unlikely to account for the inhibitory effects of SMT. In addition, the amount of thiol released from SMT over 17 h at pH 7.4 (37°C) is equivalent to the hydrolysis of less than 0.2% of the SMT present ($n = 6$). A similar value (4 ± 6%) was obtained for the hydrolysis of SMT to urea at pH 7.4. At alkaline pH, however, 45 ± 3% of SMT was converted to urea in 17 h ($n = 6$).

S-substituted isothioureas also caused dose-dependent inhibition of eNOS activity in homogenates of bovine aortic endothelial cells (Figure 3). The most potent inhibitors of iNOS activity were often also found to be the most potent inhibitors of eNOS activity. However, the order of potency was different: isopropyl-TU (**9**) > ethyl-TU (**8**) > L-NOArg (**2**) > MeArg (**1**) = SMT (**7**) > aminoethyl-TU (**25**). The potencies of ethyl-TU (**8**) and isopropyl-TU (**9**) on eNOS were reflected in their pressor effects in the anaesthetized rat. These compounds were approximately equipotent, and caused significant increases in the MAP at doses as low as 1 μg kg^{-1} (Figure 4). At the intermediate dose of 0.1 mg kg^{-1}, SMT (**7**) and aminoethyl-TU (**25**) caused pressor responses similar to those of MeArg (**1**). At higher doses (1 and 10 mg kg^{-1}), however, the pressor effects of aminoethyl-TU

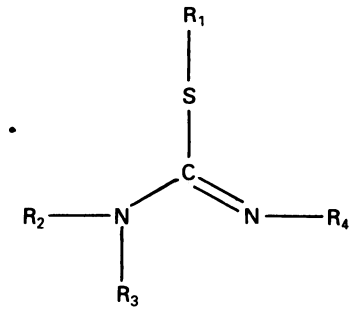

Figure 1 Generic structure of S-substituted isothioureas.

Table 2 Effect of NOS inhibitors (100 nM–1 mM) on the production of nitrite by stimulated J774.2 macrophages with increasing concentration of L-arginine (L-Arg) in the culture medium

	EC_{50} (μM)			
L-Arg (mM)	0.4*	1.4	10	30
MeArg	48	160	>1000	>>1000
SMT	6	23	160	230
Ethyl-TU	2	28	100	204
Isopropyl-TU	2	25	66	131
Aminoethyl-TU	3	35	90	270

*Normal DMEM.

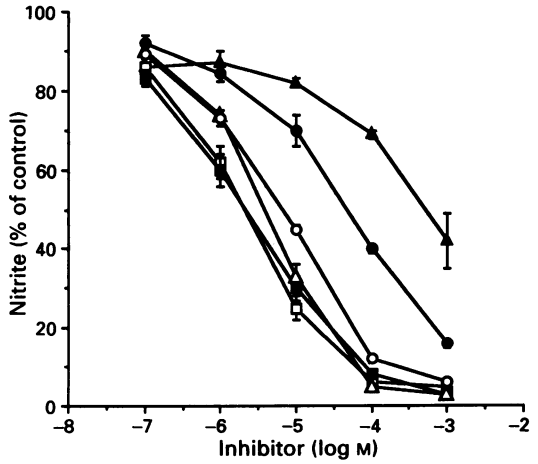

Figure 2 Effect of MeArg (●), L-NOArg (▲), SMT (○), ethyl-TU (□), aminoethyl-TU (△), and isopropyl-TU (■) when applied 6 h after lipopolysaccharide (LPS), on nitrite accumulation in the supernatant of culture J774.2 macrophages activated with LPS (1 μg ml^{-1}) for 24 h. Data (nitrite as % of control) are expressed as means ± s.e.mean of 9–12 wells from 3–4 independent experiments. Nitrite concentration in the culture medium at 24 h after LPS was 36 ± 1 μM. For abbreviations, see text.

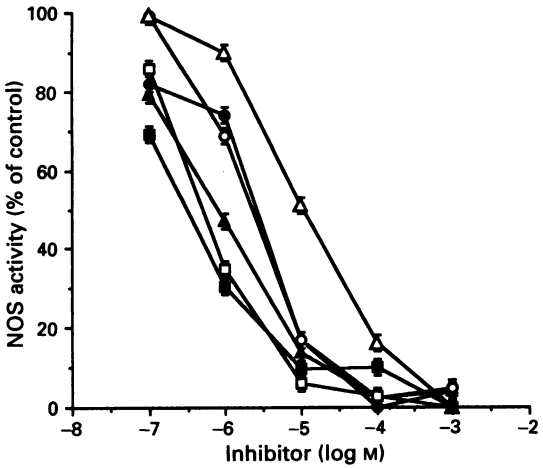

Figure 3 Effect of MeArg (●, EC_{50} = 6 μM), L-NOArg (▲, 1 μM), SMT (○, 5 μM), ethyl-TU (□, 7 μM), aminoethyl-TU (△, 12 μM), and isopropyl-TU (■, 0.5 μM) on eNOS activity, measured as formation of L-citrulline from L-arginine in homogenates of bovine endothelial cells in the presence of various co-factors and calcium (see Methods for details). Data (% of control activity, 4.12 ± 0.6 pmol mg^{-1} protein min^{-1}) are expressed as means ± s.e.mean of 5–6 experiments. For abbreviations, see text.

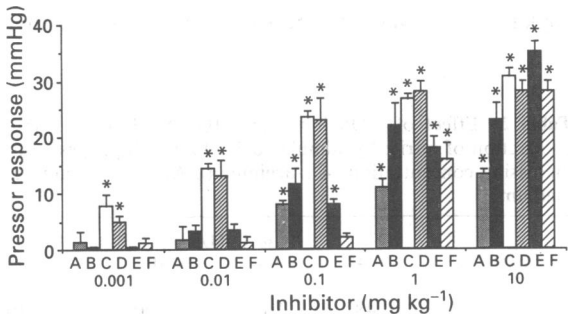

Figure 4 Changes in mean arterial blood pressure (MAP) in anaesthetized rats in response to intravenous injections of aminoethyl-TU (column A), SMT (column B), ethyl-TU (column C), isopropyl-TU (column D), MeArg (column E) and L-NOArg (column F) (0.001–10 mg kg^{-1}, i.v.). Baseline MAP prior to drug administration (128 ± 2 mmHg, n = 32) was not significantly affected by cumulative injection of vehicle (saline, n = 5). Data are expressed as means ± s.e.mean of n = 5–6 rats for each group, except SMT where n = 13. *Significant difference ($P < 0.05$) from baseline. For abbreviations, see text.

(**25**) were less than those seen with the other NOS inhibitors. Even at 10 mg kg^{-1}, the increase in the MAP seen with aminoethyl-TU (**25**) was only approximately 14 mmHg. Interestingly, L-NOArg (**2**), up to 0.1 mg kg^{-1}, failed to elicit any significant pressor response, but at higher doses it caused a pronounced pressor effect, which was similar to that caused by MeArg (**1**) (Figure 4).

Discussion

We demonstrate here that S-substituted isothioureas represent a novel class of NOS inhibitors. The EC_{50} values of some of these compounds (compounds **7**, **8**, **9** and **25**, Table 1) on iNOS activity in LPS-activated macrophages are significantly lower than those of previously reported NOS inhibitors. In addition, SMT (compound **7**, Table 1) and aminoethyl-TU (**25**) are more selective than MeArg (**1**) as inhibitors of iNOS, for they are more potent inhibitors of iNOS activity than MeArg, but less potent or equipotent to MeArg as inhibitors of eNOS activity.

Comparison of isothioureas with previously studied reference compounds, such as analogues of L-arginine, is probably the most informative estimate of their potencies. The rank orders of potency for these reference compounds on iNOS (**1**>**4**≫**2**) and eNOS (**2**>**1**>**5**) are similar to other studies. While EC_{50} values (Table 1) are somewhat higher than those reported for iNOS by some workers (Gross *et al.*, 1990; Hasan *et al.*, 1993), they are similar to those reported for cultured J774 or RAW 267.7 macrophages (Misko *et al.*, 1993; MacAllister *et al.*, 1994).

Of the compounds examined, there is a small subgroup of isothioureas that are more potent than MeArg (**1**) as inhibitors of iNOS activity in cultured macrophages: ethyl-TU (**8**) and isopropyl-TU (**9**) are approximately 24 times more potent and SMT (**7**) 6-times more potent than MeArg. These compounds have only short alkyl chains on the sulphur atom (R_1, Figure 1) and no substituents on either nitrogen. Comparison of the potencies of ethyl-, propyl-, butyl- and n-pentyl-isothioureas (**7-14**, Table 1) shows that their potencies as inhibitors of iNOS activity decline sharply if the R_1-chain extends beyond 2 carbon atoms from the sulphur. Substitution of the side chain of ethyl-TU (**8**) or SMT (**7**) (with a group X to give R_1 = $-CH_2-CH_2-X$ or R_1 = $-CH_2-X$, respectively) also results in a diminished inhibitory activity on iNOS (with the exception of aminoethyl-TU, which is 16 times more potent than MeArg). This is especially true when the substituent (X) is unsaturated (e.g. carbonyl, cyano. See compounds **22-24** in Table 1) and suggests that inductive, as well as steric, effects may influence the inhibitory activity of isothioureas on iNOS. Alkyl, amino or phenyl substituents on either one or both nitrogens of SMT substantially reduce its activity as an inhibitor of iNOS (compounds **35-45**, Table 1). Indeed, the only N-substituted compound which has a marked inhibitory effect on iNOS activity is the cyclic 2-aminothiazoline (**31**), where the imino nitrogen is linked to the sulphur. The unsaturated analogue, 2-aminothiazole (**32**), is over 10 fold weaker.

The inhibition of iNOS activity afforded by isothioureas is reduced by excess L-arginine (Table 2), demonstrating a competitive nature of the inhibition and suggesting that SMT and related compounds may occupy the L-arginine binding site of the active site of NOS.

Hydrolysis of S-substituted isothioureas can give rise to the corresponding thiols (R_1-SH) and (*inter alia*) ureas (R_2R_3N-CO-NHR_4). Ureas *per se* have no effect on iNOS activity, but some thiols do (unpublished observations). However, hydrolysis of isothioureas to the corresponding thiols is unlikely to be related to their mode of action, for (i) methanethiol (CH_3-SH, from SMT) is a weak inhibitor of nitrite accumulation, (ii) the rate of hydrolysis of some of these isothioureas to thiols is slow at pH 7.4 and (iii) there is no correlation between the inhibitory potencies of isothioureas and their ability to release the corresponding thiols (unpublished data). Although other metabolic transformation of isothioureas may occur in the cells or plasma, the rapid onset of the *in vivo* effects of isothioureas and the short incubation times used in the assay of eNOS strongly suggest that isothioureas themselves and not their metabolites are responsible for the observed inhibition of iNOS activity.

Isothioureas inhibit eNOS activity with an order of potency that is different from that seen on iNOS activity (Figure 3). The most potent inhibitors of iNOS, ethyl-TU (**8**) and isopropyl-TU (**9**), were also the most potent inhibitors of eNOS, with EC_{50} values 3–5 times lower than that of MeArg (**1**). SMT (**7**) and aminoethyl-TU (**25**), both considerably more potent than MeArg on iNOS, are only as potent as (**7**), or 6-times less potent than (**25**) MeArg on eNOS activity. Thus, when compared to MeArg, these compounds show a relative selectivity towards iNOS. In contrast, L-NOArg (**2**) preferentially inhibits eNOS activity when compared to MeArg (**1**) *in vitro* (see also: Gross *et al.*, 1990; 1991; Lambert *et al.*, 1991).

Recently, an arginine analogue, S-methylthiocitrulline, con-

taining the methylthiourea group (CH_3S-C(= NH)NH-) has been described as a potent inhibitor of NOS activity, with no apparent selectivity towards either eNOS or iNOS (Naravanan *et al.*, 1994). NOS inhibitors which are not L-arginine analogues (such as isothiourea, aminoguanidine or their derivatives) may represent a more promising approach than L-arginine analogues for the development of compounds that preferentially inhibit the activity of iNOS.

The increase in blood pressure afforded by known NOS inhibitors such as MeArg or L-NOArg is due to inhibition of eNOS activity. Thus, the pressor effects of such compounds are relevant measures of their potencies as inhibitors of eNOS *in vivo* (Gross *et al.*, 1990; Moncada *et al.*, 1991; Misko *et al.*, 1993; Hasan *et al.*, 1993). We have demonstrated that isothioureas have variable pressor effects in the anaesthetized rat (Figure 4). Ethyl-TU (**8**) and isopropyl-TU (**9**) elicit potent pressor responses (starting at doses as low as 1 $\mu g\,kg^{-1}$), whereas aminoethyl-TU (**25**) causes only modest increases in blood pressure even at doses as high as 10 $mg\,kg^{-1}$. SMT (**7**) and MeArg elicit comparable pressor responses in accord with their equivalent potencies as inhibitors of eNOS activity *in vitro*. We found that the pressor responses to L-NOArg (**2**), *in vivo*, are no greater than those to MeArg (**1**), despite L-NOArg being the more potent inhibitor of eNOS *in vitro*. This difference between the *in vivo* and *in vitro* potencies of L-NOArg vs. MeArg has also been described previously (Gross *et al.*, 1990). Such differences in the potencies of NOS inhibitors may be influenced *in vivo* by cellular uptake, distribution, metabolism, different co-factor availability etc. It is noteworthy in this respect that 7-nitro-indazole, a recently identified NOS inhibitor with selectivity towards the brain NOS isoform, is a weak pressor agent *in vivo*, but is a potent inhibitor of eNOS activity in endothelial cell homogenates with an EC_{50} similar to L-NOArg (Moore *et al.*, 1993; Babbedge *et al.*, 1993).

In the dose range of 0.01–0.1 $mg\,kg^{-1}$, MeArg (**1**), aminoethyl-TU (**25**) and SMT (**7**) elicited similar pressor effects, whereas L-NOArg (**2**) did not significantly increase blood pressure. As L-NOArg is the weakest inhibitor of iNOS activity among the compounds tested *in vivo*, a potential explanation for this phenomenon may be that a basal release of NO from iNOS may, to a small extent, contribute to the maintenance of normal blood pressure. In 'normal' animals, iNOS may be present in macrophages or in other cells and tissues (Umeda *et al.*, 1993; Morrissy *et al.*, 1994).

In conclusion, isothioureas represent a new class of NOS inhibitors. Some members of this class (ethyl-TU (**8**) and isopropyl-TU (**9**)) are potent NOS inhibitors with little isoform selectivity, whereas others (SMT (**7**) and aminoethyl-TU (**25**)) are relatively selective inhibitors of iNOS activity when compared to N^G-substituted L-arginine analogues. We have recently demonstrated that SMT has beneficial effects on haemodynamic parameters, survival rate and parameters of organ damage in rodent models of circulatory shock (Szabó *et al.*, 1994b). Thus, isothioureas may be potential tools for studying the role of NOS isoenzymes and iNOS in various pathophysiological conditions, such as circulatory shock, inflammation and cancer. In view of the accumulating evidence that supports the pathophysiological importance of iNOS in these diseases, such inhibitors may also be useful in the therapy of a variety of diseases associated with the induction of iNOS.

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