



Spontaneous rearrangement of aminoalkylisothioureas into mercaptoalkylguanidines, a novel class of nitric oxide synthase inhibitors with selectivity towards the inducible isoform

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1 The generation of nitric oxide (NO) from L-arginine by NO synthases (NOS) can be inhibited by guanidines, amidines and S-alkylisothioureas. Unlike most L-arginine based inhibitors, however, some guanidines and S-alkylisothioureas, in particular aminoethylisothiourea (AETU), show selectivity towards the inducible isoform (iNOS) over the constitutive isoforms (endothelial, ecNOS and brain isoform, bNOS) and so may be of therapeutic benefit. In the present study we have investigated the effects of AETU and other aminoalkylisothioureas on the activities of iNOS, ecNOS and bNOS.

2 AETU, aminopropylisothiourea (APTU) and their derivatives containing alkyl substituents on one of the amidino nitrogens, potentially inhibit nitrite formation by immunostimulated J774 macrophages (a model of iNOS activity) with EC₅₀ values ranging from 6–30 µM (EC₅₀ values for N^G-methyl-L-arginine (L-NMA) and N^G-nitro-L-arginine were 159 and >1000 µM, respectively). The inhibitory effects of these aminoalkylisothioureas (AATUs) were attenuated by L-arginine in the incubation medium, indicating that these agents may compete with L-arginine for its binding site on NOS.

3 The above AATUs undergo chemical conversion in neutral or basic solution (pH 7 or above) as indicated by (1) the disappearance of AATUs from solution as measured by h.p.l.c., (2) the generation of free thiols not previously present and (3) the isolation of species (as picrate and flavianate salts) from neutral or basic solutions of AATUs that are different from those obtained from acid solutions.

4 Mercaptoalkylguanidines (MAGs) were prepared and shown to be potent inhibitors of iNOS activity with EC₅₀s comparable to those of their isomeric AATUs.

5 These findings suggest that certain AATUs exert their potent inhibitory effects through intramolecular rearrangement to mercaptoalkylguanidines (MAGs) at physiological pH. Those AATUs not capable of such rearrangement do not exhibit the same degree of inhibition of iNOS.

6 In contrast to their potent effects on iNOS, some AATUs and MAGs were 20–100 times weaker than N^G-methyl-L-arginine and N^G-nitro-L-arginine as inhibitors of ecNOS as assessed by their effects on the conversion of L-arginine to L-citrulline in homogenates of bovine endothelial cells and by their pressor effects in anaesthetized rats. Thus mercaptoalkylguanidines represent a new class of NOS inhibitors with preference towards iNOS.

7 AETU and mercaptoethylguanidine (MEG), when given as infusions, gave slight decreases in MAP in control rats. However, infusions of AETU or MEG to endotoxin-treated rats caused an increase in MAP and restored 80% of the endotoxin-induced fall in MAP.

8 High doses of MEG (30–60 mg kg⁻¹) caused a decrease in MAP of normal rats. This depressor effect may be a consequence of the *in vivo* oxidation of MEG to the disulphide, guanidinoethylidysulphide (GED), which caused pronounced, transient hypotensive responses in anaesthetized rats and caused endothelium-independent vasodilator responses in precontracted rat aortic rings *in vitro*.

9 In some cases, slight differences were observed in the activities of AATUs and the corresponding MAGs. These may be explained by the formation of other species from AATUs in physiological media. For example, AETU can give rise to small amounts of the potent ecNOS inhibitor, 2-aminothiazoline, in addition to MEG. This may account for the differences in the *in vitro* and *in vivo* effects of AETU and MEG.

10 In conclusion, the *in vitro* and *in vivo* effects of AETU and related aminoalkylisothioureas can be explained in terms of their intramolecular rearrangement to generate mercaptoalkylguanidines, a novel class of selective inhibitors of iNOS.

Keywords: Nitric oxide; mercaptoalkylguanidines; isothioureas; blood pressure; shock; isoform-selective inhibition; endotoxin; vasoconstriction; vasodilatation; EDRF

Introduction

Nitric oxide (NO), a mediator of many physiological and pathophysiological processes, is produced by the oxidation of L-arginine by a family of isoenzymes—nitric oxide synthases (NOS) (see: Nathan, 1992; Marletta, 1993 for reviews). NO

derived from NOS located in the central and peripheral nervous system (brain NOS, bNOS) has important physiological roles as a neurotransmitter and as a potential mediator of the metabolism/blood flow coupling in the brain (Snyder & Bredt, 1992). It may also be involved in the pathogenesis of neuroinjury (Dawson *et al.*, 1991; Garthwaite, 1991). NO from the constitutive endothelial NOS (ecNOS) is involved in, *inter alia*, the regulation of blood pressure and blood flow to organs (see:

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Vane, 1994 for review). Reduced production of NO by eNOS can result in vasoconstriction, increased blood pressure and reduced blood flow to organs and is implicated in the pathophysiology of a number of vascular and non-vascular diseases (Vane 1994; Dinerman *et al.*, 1993).

Enhanced formation of nitric oxide (NO) following the induction of a distinct isoform of NOS (inducible NOS, iNOS) by pro-inflammatory agents, such as endotoxin (bacterial lipopolysaccharide, LPS), interleukin-1 β and γ -interferon (IFN), in a variety of cells including macrophages (see: Nathan, 1992; Xie & Nathan, 1994 for reviews) has been implicated in the pathogenesis of various forms of circulatory shock and inflammation (see: Nathan, 1992; Szabo & Thiemermann, 1993 for reviews). The pathophysiological importance of over-production of NO by iNOS suggests that inhibitors of iNOS have therapeutic potential, in particular those that do not affect the protective and physiological roles of eNOS.

The generation of nitric oxide (NO) by NOS can be inhibited by analogues of the substrate, L-arginine. However, the most commonly used inhibitors of NOS, namely N^G-methyl-L-arginine (L-NMA), N^G-nitro-L-arginine (L-NOARG) and its methyl ester, L-NAME, inhibit eNOS at least as strongly as they inhibit iNOS (Gross *et al.*, 1990; 1991; Lambert *et al.*, 1991).

Recently, compounds that are not amino acids, such as guanidines (Hasan *et al.*, 1993; Macallister *et al.*, 1994), S-alkylisothioureas (Szabó *et al.*, 1994a; Garvey *et al.*, 1994; Southan *et al.*, 1995b) and amidines (Southan *et al.*, 1995a) have also been reported to inhibit NOS. Unlike most L-arginine based inhibitors, however, aminoguanidine (Misko *et al.*, 1993), some S-alkyl-isothioureas (Garvey *et al.*, 1994; Southan *et al.*, 1995b; Nakane *et al.*, 1995), and some amidines (Southan *et al.*, 1995a) have been reported to exhibit selectivity towards iNOS and so may be of therapeutic benefit (Corbett *et al.*, 1992; Szabó *et al.*, 1994a; Wu *et al.*, 1995; Worrall *et al.*, 1995). Among the S-substituted isothioureas, S-aminoethylisothiourea (AETU) shows a marked selectivity towards iNOS when studied in rodent enzyme systems (Southan *et al.*, 1995b). We have, therefore, investigated the effects of AETU and other aminoalkylisothioureas on the activities of iNOS and eNOS. In view of the chemical nature of AETU, we have also investigated whether AETU itself, or some other species, is the mediator of these inhibitory effects. Here, we demonstrate that the *in vivo* and *in vitro* pharmacological actions of AETU and related aminoalkylisothioureas can be explained by their rearrangement to yield mercaptoalkylguanidines (MAGs), a new class of potent inhibitors of NOS.

Methods

Measurement of thiols

Nitroprusside test (qualitative): a solution of the compound to be tested (~1 mg in 1 ml water) was brought to around pH 8–10 with a drop of saturated sodium carbonate solution. A drop of a freshly prepared solution of sodium nitroprusside (2% sodium nitroferrocyanide(III) dihydrate) was then added. A positive test for free thiol (sulphurhydriyl, -SH) was indicated by the immediate formation of a red/purple solution. Although hydrolysis of isothioureas to alkylthiols and urea can occur at the pH of the sodium carbonate solution used, this is slow compared to the instantaneous colour formed with thiols. Isothioureas such as S-methyl and S-ethyl-isothioureas gave negative tests. Some aminoalkylisothioureas give positive tests due to their rearrangement at the pH used for this test (see: Discussion).

Kinetics: a saturated solution of 'Aldrithiol-2^D (2,2'-dipyridyl disulphide, 100 μ l; see: Grasetti & Murray, 1967) was added to solutions of AATUs or other compounds in distilled water (50 μ l, 1 mM, pH ~ 5.5–6) in 96 well plates. Phosphate buffer (50 μ l, 100 mM pH 7.0, 7.4, 7.7, 8.0 or 8.9) was added and the

absorbance at 343 nm measured immediately (3 min) and subsequently at 25, 90, 200 and 415 min and after 24 h with a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). The background signal for Aldrithiol-2 did not change over 24 h and was subtracted from all readings. The corrected absorbances were compared to cysteine standards and expressed as % of signal for 1 mM cysteine. Cysteine gave quantitative reduction of Aldrithiol-2, based on the extinction coefficient for the reduction product, 2-thiopyridone (7060 at pH 7.2; Grasetti & Murray, 1967). For aminoalkylisothioureas this is equivalent to % of theoretical yield of liberated thiol (see Results and Discussion).

High performance liquid chromatography

High performance liquid chromatography (h.p.l.c.) quantitation of AATUs was performed on a Varian 5000 LC system (Varian Instruments, Sugar Land, Texas, U.S.A.) using a weak cation exchanger (Universal Cation, 7 μ , 100 mm $\times$ 4.6 mm i.d., Alltech, Avondale, PA, U.S.A.) and eluting isocratically with 28 mM potassium acetate/HCl buffer (pH 2.5) at a flow rate of 1.2 ml min⁻¹. Eluting peaks were quantitated by u.v. absorbance at 214 nm. Some typical elution times were: compound No. 5 (refer to Table 1), 2.1 min; No. 6, 2.3; No. 9, 1.9; No. 10, 1.9; No. 11, 2.1; No. 2, 2.4; No. 14, 2.2 and No. 16, 3.0. The simultaneous analysis of AATUs and MAGs could not be performed because (i) the extreme basicity of MAGs prevents their elution from this column under these or more severe conditions and (ii) strongly alkaline conditions, which are often employed to elute guanidines, would destroy AATUs.

Cell culture

The mouse macrophage cell line J774.2 was cultured in Dulbecco's modified Eagle's medium (DMEM) with 4 $\times$ 10⁻³ M L-glutamine and 10% foetal calf serum as described (Szabó *et al.*, 1994a,b). Cells were cultured in 96-well plates with 200 μ l culture medium until they reached 60–80% confluence. To induce iNOS, fresh culture medium containing *E. coli* LPS (10 μ g ml⁻¹) and murine γ -interferon (IFN, 50 u ml⁻¹) was added. Nitrite accumulation in the cell culture medium (in the absence or presence of various inhibitors) was measured after 24 h. Unless stated otherwise, NOS inhibitors were added 6 h after LPS/IFN in order to prevent interference with the process of iNOS induction (see: Szabó *et al.*, 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 production

Nitrite production, an indicator of NO synthesis, was measured in the supernatant of J774.2 macrophages as previously described (Szabó *et al.*, 1994b). Nitrite was measured by adding 100 μ l of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) to 100 μ l samples of cell culture medium. The optical density at 550 nm (OD₅₅₀) was measured by using the Spectramax microplate reader. Nitrite concentrations were calculated by comparison with OD₅₅₀ of standard solutions of sodium nitrite prepared in culture medium.

Cell respiration

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan (Szabó *et al.*, 1994b). Cells in 96-well plates were incubated with MTT (0.2 mg ml⁻¹) for 60 min at 37°C. Culture medium was removed by aspiration and the cells solubilized in dimethylsulphoxide (100 μ l). The extent of reduction of MTT to formazan within cells was quantitated by mea-

surement of OD₅₅₀ using the Spectramax microplate reader. The inhibitory effects on nitrite production presented in the figures and tables were not accompanied by significant decreases in cell viability, except as noted otherwise.

Nitric oxide synthase assay

Three cell/tissue types expressing high levels of the various NOS enzymes were used in our studies to investigate the effect of NOS inhibitors on the activity of various NOS isoforms. Calcium-dependent conversion of L-arginine to L-citrulline in cell homogenates obtained from the scraped intimal surface of fresh bovine aortae served as a model of eNOS activity (Southan *et al.*, 1995b); calcium-dependent L-arginine-L-citrulline conversion in homogenates of rat brain served as model of bNOS activity (Wu *et al.*, 1995) and calcium-independent conversion of L-arginine to L-citrulline in homogenates of lungs obtained from rats treated with *E. coli* LPS (15 mg kg⁻¹, i.v. for 180 min) served as model of iNOS activity (Szabó *et al.*, 1994a).

Tissues/cells were homogenized in a buffer composed of 50 mM Tris-HCl, 0.1 mM EDTA and 1 mM phenylmethylsulphonyl fluoride (pH 7.4) on ice using a Tissue Tearor 985-370 homogenizer (Biospec Products, Racine, WI, U.S.A.). Conversion of [³H]-L-arginine to [³H]-L-citrulline was measured in the homogenates as described (Southan *et al.*, 1995b). Briefly, homogenates (30 µl) were incubated in the presence of [³H]-L-arginine (10 µM, 5 kBq/tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 µM) and calcium (2 mM) for 20 min at 22°C for eNOS and bNOS measurements. This assay has been previously verified for the measurement of the activity of various NOS isoforms, and the production of L-citrulline from L-arginine by NOS was found to be linear for 20–30 min. For iNOS measurements, calcium was omitted and replaced with EGTA (2 mM). Reactions were stopped by dilution with 0.5 ml of ice cold HEPES 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 [³H]-L-citrulline activity was measured by a Wallac scintillation counter (Wallac, Gaithersburg, MD, U.S.A.).

NOS activities are expressed as percent of total activity in the absence of NOS inhibitors. The absolute values of NOS activities in the cell homogenates were 1.8 ± 0.01 pmol mg⁻¹ min⁻¹ for eNOS, 1.9 ± 0.2 pmol mg⁻¹ min⁻¹ for bNOS and 1.2 ± 0.04 pmol mg⁻¹ min⁻¹ for iNOS.

Haemodynamic measurements

The pressor effects of known NOS inhibitors were used to assess the ability of the drug to inhibit eNOS activity *in vivo*. Male Wistar rats (Charles River Laboratories, Wilmington, MA, U.S.A.) were anaesthetized with sodium thiopentone (120 mg kg⁻¹, i.p.) and instrumented as described (Southan *et al.*, 1995b). The trachea was cannulated to facilitate respiration and temperature was maintained at 37°C with a homeothermic blanket. The right carotid artery was cannulated and connected to a pressure transducer for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate which were digitalized with a MacLab A/D converter (AD Instruments, Milford, MA, U.S.A.) and stored and displayed on a Macintosh personal computer. The left femoral vein was 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 (0.1–10 mg kg⁻¹, i.v.) in a cumulative fashion. The separate bolus injections were performed in every 5 min. Dose-response curves were performed once in each animal and each animal received only one NOS inhibitor.

In animals subjected to endotoxin shock, the pressor effect of a NOS inhibitor is a good indicator of the ability of the agent to inhibit iNOS. The effects of infusions of selected NOS inhibitors into control animals and into rats subjected to en-

dotoxin shock then allows the effects of the agents on iNOS and eNOS *in vivo* to be compared. In these studies, rats were injected with *E. coli* LPS (15 mg kg⁻¹, i.v.) at time 0. At 90 min, then saline was infused at a rate of 0.2 ml kg⁻¹ for a further 90 min (vehicle-treated LPS-rats). Alternatively at 90 min, AETU, MEG or ATZ was injected (at 10 mg kg⁻¹, i.v. in 0.1 ml kg⁻¹ saline) and then infused (30 mg kg⁻¹ h⁻¹ in 0.2 ml kg⁻¹) for a further 90 min (NOS-inhibitor treated LPS-rats).

In the control groups of rats, animals were injected with vehicle (saline, 0.1 ml kg⁻¹, i.v.) at time 0. At 90 min, AETU, MEG or ATZ was injected (at 10 mg kg⁻¹, i.v. in 0.1 ml kg⁻¹ saline) and then infused (30 mg kg⁻¹ h⁻¹ in 0.2 ml kg⁻¹) for a further 90 min (NOS-inhibitor treated control-rats).

Organ bath experiments

Thoracic aortae from rats were cleared of adhering periadventitial fat and cut into rings of 3–4 mm width. Endothelium was removed from some of the rings by gently rubbing the intimal surface. Lack of an acetylcholine-induced relaxation was taken as evidence that endothelial cells had been removed. The rings were mounted in organ baths (5 ml) filled with warmed (37°C), oxygenated (95% O₂/5% CO₂) Krebs solution (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25 and glucose 11.7, in the presence of indomethacin (10 µM). Isometric force was measured with isometric transducers (Kent Scientific Corp. Litchfield, CT, U.S.A.), digitalized using a MacLab A/D converter (AD Instruments, Milford, MA, U.S.A.) and stored and displayed on a Macintosh personal computer. A tension of 1 g was applied and the rings were equilibrated for 60 min, changing the Krebs solution every 15 min.

Concentration-response curves to noradrenaline (10⁻⁹–10⁻⁵ M) in the presence of NOS inhibitors (100 µM, 30 min treatment) were obtained in endothelium-denuded aortic rings taken from either control rats or rats injected with LPS (15 mg kg⁻¹, i.v.) and killed 180 min later.

The effect of pretreatment with NOS inhibitors (10⁻⁶–10⁻⁴ M 30 min treatment) on endothelium-dependent relaxations elicited by acetylcholine (10⁻⁹–10⁻⁶ M) was investigated in intact aortic rings (obtained from control rats) after pre-contraction with noradrenaline (10⁻⁷ M).

Materials

Acetylcholine chloride, aminoguanidine, bacterial lipopolysaccharide (*E. coli*, serotype No. 0127:B8), calmodulin, L-glutamine, MTT, NADPH, N^G-nitro-L-arginine (L-NOARG), N^G-nitro-L-arginine methyl ester (L-NAME), noradrenaline hydrochloride, sodium nitrite, sodium nitrate and Dowex 50W anion exchange resin were obtained from Sigma (St. Louis, MO, U.S.A.). DMEM, RPMI and foetal calf serum were from Gibco (Grand Island, NY, U.S.A.). N^G-methyl-L-arginine monoacetate (L-NMA) was from Calbiochem (La Jolla, CA, U.S.A.). Sodium thiopentone was obtained from Abbot Laboratories (Chicago, IL, U.S.A.). Murine IFN was from Genzyme (Cambridge, MA, U.S.A.). Tetrahydrobiopterin was obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.). S-methyl- and S-ethyl-isothioureas, were obtained from Aldrich (St. Louis, MO, U.S.A.). [2,3,4,5-³H]-L-arginine hydrochloride was obtained from DuPont/NEN (Boston, MA, U.S.A.).

Synthesis of aminoalkylisothioureas and mercaptoalkylguanidines

Aminoalkylisothioureas (AATUs) were prepared by refluxing the appropriate thiourea with appropriate bromoalkylamine hydrobromide in isopropanol for 0.5–1 h. Reagent concentrations were chosen to encourage precipitation of the dihydrobromide (.2HBr) salts upon cooling of the reaction mixture (Doherty *et al.*, 1957; Southan *et al.*, 1995b).

MEG sulphate was prepared by the reaction of mercap-

toethylamine with S-methylisothiourea in methanol at room temperature. In addition, solutions of mercaptoethylguanidine (MEG), mercaptopropylguanidine (MPG) and other MAGs were prepared from aqueous solutions of AETU.2HBr, APTU.2HBr and appropriate AATU salts respectively, by the addition of a molar equivalent of sodium hydroxide (1 M solution) or a quantity sufficient to maintain the pH at 7.0–7.4 for 20 min (Khym *et al.*, 1958; Shapiro *et al.*, 1963). S-methyl-mercaptoethylguanidine (2-(methylthio) ethylguanidine) was prepared (as the sulphate salt) by reacting 2-(methylthio) ethylamine with S-methylisothiuronium sulphate in 90% aqueous methanol overnight at room temperature. The methanol was evaporated under reduced pressure and the remaining solution filtered and evaporated further to leave an oil that crystallised overnight. The solid was recrystallised from methanol/ether to give opaque colourless crystals of the product. S-ethylmercaptoethylguanidine sulphate was prepared from 2-(ethylthio) ethylamine in a similar way.

The disulphide dimer of MEG, guanidinoethyl-disulphide (GED), was prepared by drawing filtered air through a solution of MEG (prepared from AETU as above) for 45 h (Hino *et al.*, 1966). The solution was evaporated to dryness and the solid vacuum desiccated over sodium hydroxide, before being recrystallised from methanol as colourless needles.

4-Bromobutylamine.HBr, used in the synthesis of S-(4-aminobutyl)isothiourea was prepared by slowly adding thionyl bromide to a solution of 4-aminobutanol in dry chloroform on ice.

Elemental analyses (C,H,N) of synthesized compounds (performed by Galbraith Laboratories, Inc, Knoxville, TN, U.S.A.) showed % composition to be within 0.6% of theoretical. h.p.l.c. analysis (see above) showed one peak (other than sulphate of bromide) for each compound. Melting points were consistent with literature values, where available.

Statistical evaluation

All values in the figures and text are expressed as mean $\pm$ standard error of the mean (s.e.mean) of *n* observations. For each agent tested, 6–15 wells from at least 2 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

Effects of aminoalkylisothioureas (AATUs) and mercaptoalkylguanidines (MAGs) on nitrite production in immunostimulated macrophages

The concentration of nitrite in the medium of the J774.2 macrophages following immuno-stimulation with γ -interferon (IFN) and lipopolysaccharide (LPS) reached $67 \pm 3 \mu\text{M}$ after 24 h. No nitrite could be detected in the first 6 h or in the absence of immuno-stimulation. In the presence of aminoalkylisothioureas (AATUs) or mercaptoalkylguanidines (MAGs, see Figure 1 and Table 1 for general structures) there was a dose-dependent inhibition of nitrite formation (Figure 2a).

In this model of iNOS activity, AETU (compound No. 4, Table 1), APTU (No. 5) and their derivatives (Nos. 9–17, Table 1) were, in general, much more potent than L-NMA, L-NOARG or aminoguanidine (Figure 2a), which showed EC_{50} values similar to those previously reported for macrophages (see: Southan *et al.*, 1995b). Substituents on the amidino nitrogens appeared to reduce the potencies of AATUs: for instance AETU > N-methyl-AETU > N,N'-dimethyl-AETU (No. 4 > No. 10 > No. 12, Table 1), although for mono N-substituted derivatives (Nos. 9, 10, 11 and 14, 15) the differences are relatively small. Substitution of the amino nitrogen of

Table 1 EC_{50} values for the inhibition of nitrite production by stimulated J774 macrophages by aminoethylisothioureas (*AATUs), mercaptoalkylguanidines (MAGs) and other agents

No.	Compound	X	R*	R*	R**	EC_{50} (μM)
1	N ^G -methyl-L-arginine (L-NMA)	NH	-(CH ₂) ₃ CH(NH ₂)CO ₂ H	-CH ₃	H	159
2	N ^G -nitro-L-arginine (L-NOARG)	NH	-(CH ₂) ₃ CH(NH ₂)CO ₂ H	-NO ₂	H	~1000
3	Aminoguanidine	NH	-NH ₂	H	H	120
4	Aminoethyl-TU (AETU)	S	-CH ₂ CH ₂ NH ₂	H	H	14
5	Aminopropyl-TU (APTU)	S	-CH ₂ CH ₂ CH ₂ NH ₂	H	H	8
6	Aminobutyl-TU (ABTU)	S	-CH ₂ CH ₂ CH ₂ CH ₂ NH ₂	H	H	51
7	S-(dimethylaminoethyl)-TU	S	-CH ₂ CH ₂ N(CH ₃) ₂	H	H	205
8	S-(dimethylaminopropyl)-TU	S	-CH ₂ CH ₂ CH ₂ N(CH ₃) ₂	H	H	340
9	N-methyl-AETU	S	-CH ₂ CH ₂ NH ₂	-CH ₃	H	29
10	N-ethyl-AETU	S	-CH ₂ CH ₂ CH ₂ NH ₂	-CH ₂ CH ₃	H	27
11	N-allyl-AETU	S	-CH ₂ CH ₂ NH ₂	-CH ₂ CH=CH ₂	H	24
12	N,N'-dimethyl-AETU	S	-CH ₂ CH ₂ NH ₂	-CH ₃	-CH ₃	515
13	N,N'-ethylene-AETU	S	-CH ₂ CH ₂ NH ₂	-CH ₂ CH ₂ -**	H	96
14	N-methyl-APTU	S	-CH ₂ CH ₂ CH ₂ NH ₂	-CH ₃	H	14
15	N-ethyl-APTU	S	-CH ₂ CH ₂ CH ₂ NH ₂	-CH ₂ CH ₃	H	13
16	N,N'-dimethyl-APTU	S	-CH ₂ CH ₂ CH ₂ NH ₂	-CH ₃	-CH ₃	350
17	N,N'-ethylene-APTU	S	-CH ₂ CH ₂ CH ₂ NH ₂	-CH ₂ CH ₂ -**	H	43
18	Mercaptoethylguanidine (MEG)	NH	-CH ₂ CH ₂ SH	H	H	13
19	Mercaptopropylguanidine (MPG)	NH	-CH ₂ CH ₂ CH ₂ SH	H	H	15
20	S-methyl-MEG	NH	-CH ₂ CH ₂ SCH ₃	H	H	80
21	S-ethyl-MEG	NH	-CH ₂ CH ₂ SCH ₂ CH ₃	H	H	328
22	2-Aminothiazoline (ATZ)	S	-CH ₂ CH ₂ -***	H	H	187
23	Guanidylethylenedisulphide (GED)	NH	< >	H	H	6
24	Hydroxyethylguanidine	NH	-CH ₂ CH ₂ OH	H	H	>1000
25	Aminobutylguanidine (agmatine)	NH	-CH ₂ CH ₂ CH ₂ CH ₂ NH ₂	H	H	>1000
26	(S-methylthioethyl)isothiourea	S	-CH ₂ CH ₂ SCH ₃	H	H	230

*Refer to Figure 1 for positions of R groups; **R' and R'' are replaced by ethylene bridge indicated to form a 5-membered (imidazolidine) heterocycle; ***R and R' are replaced by ethylene bridge indicated to form a 5-membered (thiazoline) heterocycle; < >, R = -(CH₂)₂SS(CH₂)₂NHC(=NH)NH₂.

AETU with two methyl groups (as in S-(dimethylaminoethyl)-TU (No. 7) and S-(dimethylaminopropyl)-TU (No. 8)) caused a large reduction in the potency.

While the potency of MEG (No. 18) is similar to that of AETU (No. 4), S-substitution of MEG appears to reduce its potency against nitrite accumulation (MEG > S-methyl-MEG (No. 20) > S-ethyl-MEG (No. 21)). However, the results on iNOS activity in lung homogenates suggest otherwise (see below).

The oxygen analogue of MEG, 2-hydroxyethylguanidine (No. 24), has very little effect on nitrite production, while the isothiurea analogue of S-methyl-MEG (No. 20), i.e. S-(methylthioethyl)isothiurea (No. 26), is less effective than S-methyl-MEG (Table 1).

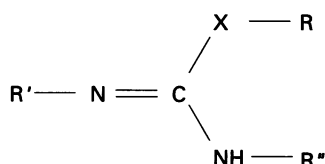


Figure 1 Structure of aminoalkylguanidines (X=NH; compounds Nos. 4–17 in Table 1) and mercaptoalkylguanidines (X=S; compounds Nos. 18–21 in Table 1). R, R' and R'' are defined in Table 1.

The inhibitory effects of these agents were generally not associated with inhibition of mitochondrial respiration up to the concentration of 1 mM. However, GED was found to inhibit mitochondrial respiration in macrophages at 100 μM (by $23 \pm 3\%$, $P < 0.05$, $n = 9$). Direct cytotoxicity, however, cannot be responsible for its potent inhibitory effect on nitrite production, which was already significant at 1 μM and was approximately 60% at 10 μM , concentrations at which GED did not inhibit mitochondrial respiration.

Attenuation of the inhibitory effect of AATUs and MAGs by L-arginine

The effect of AATUs and of MAGs on nitrite production by macrophages was reduced in a dose-dependent manner by increasing concentrations of L-arginine in the incubation medium (Table 2). This suggests that MAGs and AATUs may compete with L-arginine for the substrate binding site of NOS.

Production of free thiols from AATUs in solution

Dissolution of AETU (No. 4) or APTU (No. 5) (dihydrobromide salts) in distilled water gave solutions with a pH of 5.5–6, equivalent to that of distilled water. Addition of picric

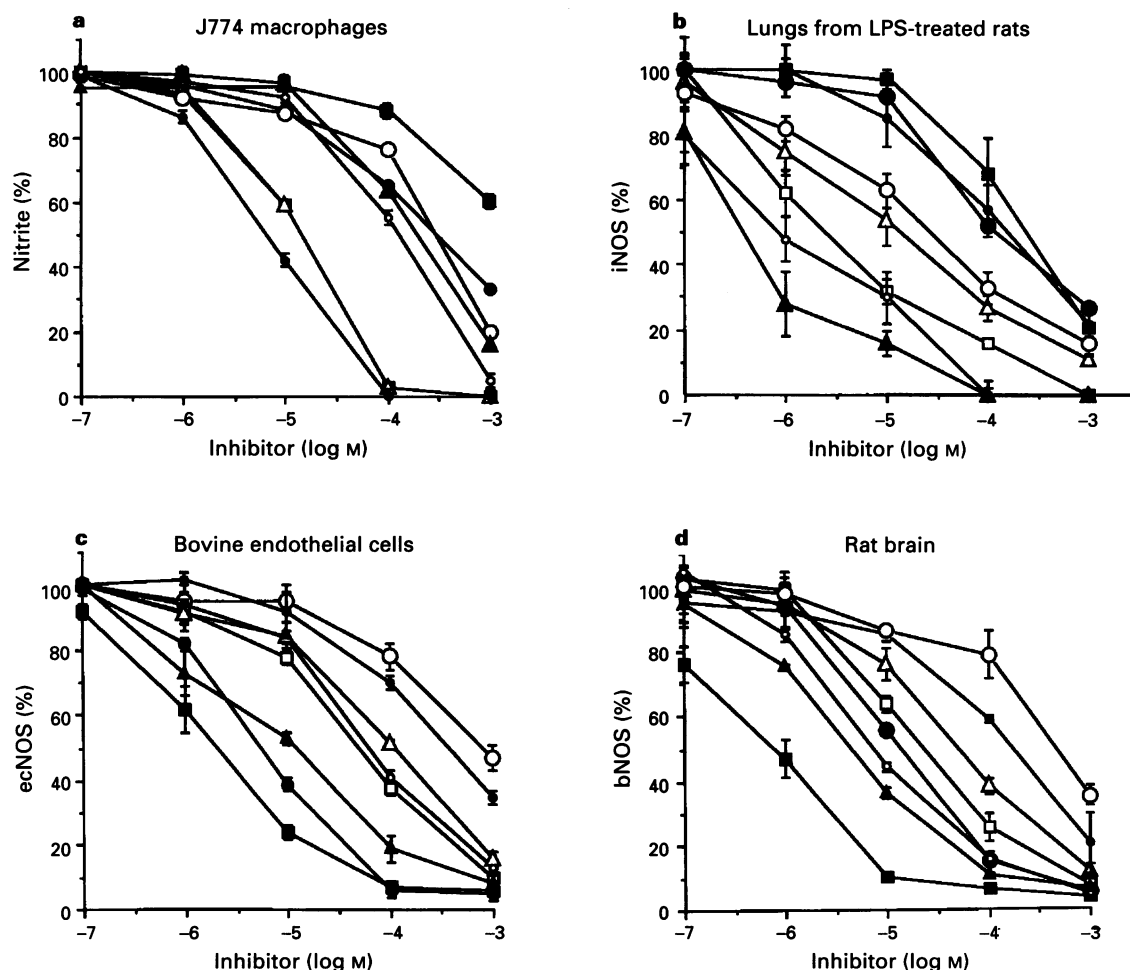


Figure 2 Effect of L-NMMA (●), L-NOARG (■), AETU (□), MEG (△), S-methyl-MEG (○), S-ethyl-MEG (○), GED (●) and ATZ (▲), on (a) nitrite accumulation in the supernatant of cultured J774.2 macrophages activated with LPS ($10 \mu\text{g ml}^{-1}$) and INF (50 u ml^{-1}) for 24 h (drugs were applied 6 h after LPS), (b) iNOS activity in homogenates of lungs taken from LPS-treated rats, (c) ecNOS activity in homogenates of bovine endothelial cells, (d) bNOS activity in homogenates of rat brain. In (b), (c) and (d), NOS activity was measured as formation of L-citrulline from L-arginine in the presence of appropriate co-factors. Data (expressed as % of control) represent means $\pm$ s.e.mean of 9–12 determinations from 3–4 independent experiments. Where not shown, error bars are contained within the symbols.

acid, either immediately or after acidification with acetic acid (pH 3), caused the precipitation of the yellow picrate salts (melting points: 224°C and 186–7°C, respectively). When the solutions were first brought to neutral pH (7–7.5) with sodium hydroxide solution, addition of picric acid precipitated salts with melting points (and appearances) that were substantially different (159–161°C and 148–151°C respectively). However, picrates isolated from solutions of S-(dimethylaminoethyl)-TU(7) and S-(dimethylaminopropyl)-TU (No. 8) had melting points of 243–5° and 235°C respectively at all three pH values. The same pH dependence was also true for the flavianate salts of AETU and APTU isolated from aqueous solutions in the same way.

In addition, AETU (No. 4) and APTU (No. 5) (and other AATUs, Nos. 9–17) gave positive nitroprusside tests, indicative of the presence of free thiol (sulphydryl) groups, while S-(dimethylaminoethyl)-TU (No. 7) and S-(dimethylaminopropyl)-TU (No. 8) did not. Using the reagent 2-dipyridyl disulphide (2,2'-dithiopyridine, Aldrithiol-2) no thiol was detected in acidic (pH 3) solutions of any of the AATUs, except for a small amount (5%) from AETU after 1 h. In neutral or mildly basic solution (pH 7–9), however, variable amounts of thiol were seen for AETU, APTU and their derivatives, but not for aminobutyl-TU (No. 6), S-(dimethylaminoethyl)-TU (No. 7) and S-(dimethylaminopropyl)-TU (No. 8), even after 12 h (Figure 3).

Analysis of solutions of these compounds by h.p.l.c. showed the loss of peaks for AETU (No. 4) and APTU (No. 5) when made weakly basic (pH 7–8), but not for aminobutyl-TU (No. 6), S-(dimethylaminoethyl)-TU (No. 7) or S-(dimethylaminopropyl)-TU (No. 8). The rate of generation of thiol from AATUs increased with pH (Figure 3) of the solution, while substitution of the amidino nitrogens of APTU (compounds Nos. 14–17) reduced the rate. AETU produced thiol most rapidly (95% conversion within 3 min and no subsequent increase) and the generation of free thiols was the slowest in the case of N,N'-dimethyl-APTU (No. 16). In general, the rate of disappearance of the AATUs (measured by h.p.l.c., data not shown) correlated with the rate of formation of the thiol for those compounds analysed (Nos. 6, 9–15, 17), although at pH 8.9, the h.p.l.c. signal of aminobutyl-TU (No. 6) diminished at a rate of 8% every 15 min without generating any thiol. At elevated pH, hydrolysis of isothioureas to give mercaptoalkylamines (containing a free thiol) and urea may occur. However, under the same conditions as above (Figure 3), the conversion of S-methyl- and S-ethylisothioureas to free thiol was less than 4% at pH values 7–8 after 24 h and 6–9% at pH 8.9 (Figure 3). Thus, in the context of these experiments, the rate of hydrolysis of isothioureas is negligible. It is clear, however, that the rate of generation of the free thiol varies between AATUs tested.

EC₅₀ values shown in Table 1 are for standard solutions prepared in distilled water (pH 5.5–6). When the standards were prepared from stock solutions of AATUs neutralized (pH ~7.4) with sodium hydroxide, small differences in EC₅₀ values were observed in some cases. These EC₅₀ values for

compounds in neutral solution (pH 7.2–7.4) were 14 µM (for AETU, No. 4), 15 µM (APTU, No. 5), 18 µM (N-Me-APTU, No. 14), 570 µM (di-Me-AETU, No. 12) and 510 µM (di-Me-APTU, No. 16). The pH of the medium for the duration of the 24 h incubation was 7.4 in all cases.

Inhibitory effect of AATUs and MAGs on the activities of iNOS, ecNOS and bNOS in cell homogenates

As the inhibitory potency of NOS inhibitors in whole cell systems may be affected by a number of factors (including cellular uptake), the effect of AATUs and MAGs on the activities of iNOS, ecNOS and bNOS were compared in various cell/tissue homogenates in the presence of appropriate co-factors. AETU (No. 4) and APTU (No. 5) were extremely potent at inhibiting the conversion of L-arginine to L-citrulline by iNOS when compared to L-NMA, L-NOARG or aminoguanidine (Table 3). S-methyl-MEG (No. 20) was as potent as MEG (No. 18), despite its relatively weaker inhibitory effects in intact cells. The same was true for ATZ (No. 22) and, to a lesser extent, for S-ethyl-MEG (No. 19, compare Tables 1 and 3). ATZ was over 200 times more effective against iNOS in

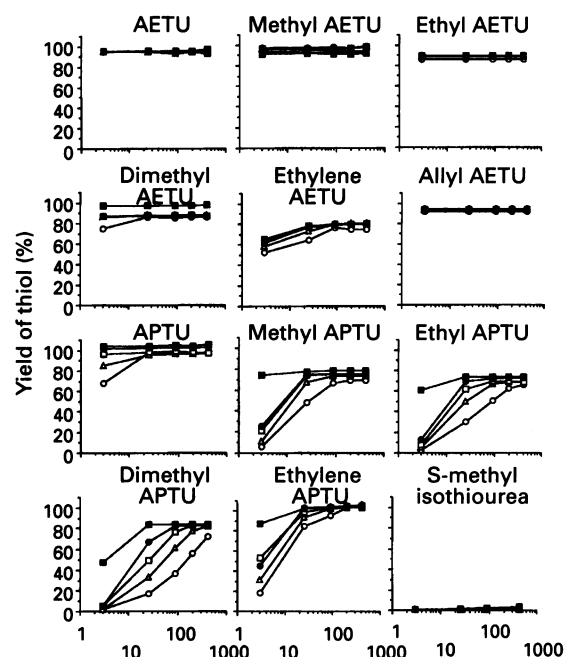


Figure 3 Time courses for the generation of free thiol (sulphurhydryl, -SH) from AATUs in phosphate buffer at pH 7.0 (○), 7.4 (△), 7.7 (□), 8.0 (●) and 8.9 (■). Data are expressed as percentage of theoretical maximum yield (means ± s.e. mean of 6 wells). Error bars are contained within the symbols.

Table 2 EC₅₀ values of AATUs and related compounds for their inhibition of nitrite formation by J774 macrophages in the presence of increasing concentrations of L-arginine

No.	Compound	L-Arginine (mM)			
		0.4	1.4	10	30
4	AETU	14	27	140	405
5	APTU	7	24	110	230
9	N-methyl-AETU	16	37	185	395
14	N-methyl-APTU	26	130	425	40%
20	S-methyl-MEG	80	230	625	32%
23	GED	5	21	103	390

homogenates than in intact cells. Conversely, the disulphide dimer of MEG, GED (No. 23), was far less effective against iNOS in the homogenate than against NO production by intact cells.

MEG (No. 18), whether prepared by neutralization of a solution of AETU or by an independent synthetic route (see Methods), was slightly less potent than AETU (No. 4) against iNOS in homogenates of lung obtained from endotoxin-treated rats (Figure 2b). A similar observation was made when comparing the effects of MPG (No. 19) and APTU (No. 5) (Table 3). These differences were even smaller in the intact cells.

The effects of AATUs and MAGs on ecNOS activity and bNOS activity were also compared in homogenates of bovine endothelial cells (ecNOS) and rat brain (bNOS) (Table 3). Their effects appeared similar against both constitutive isoforms (Figure 1c, d), except that S-methyl MEG (No. 20) is more potent against bNOS activity, while L-NMA is more potent against ecNOS activity. Even though MEG (No. 18) (and AETU (No. 4)) and its S-alkylated derivatives (No. 20, No. 21) were more potent than L-NMA against iNOS activity, they were found to be weaker than L-NMA and L-NOARG

against ecNOS activity (Figure 2c; Table 3). APTU and its N-methyl derivative, however, showed little discrimination between constitutive and inducible isoforms.

Pressor effects of AATUs and MAGs

The pressor responses of the drugs in anaesthetized rats, a reflection of their effects on ecNOS *in vivo*, are in general agreement with their inhibitory effects on ecNOS *in vitro*. The data shown in Figure 4 depicts the sustained changes in MAP measured after 5 min in response to bolus i.v. injection of the compound. L-NMA, L-NOARG, MPG and ATZ (No. 22), all potent against ecNOS *in vitro*, all gave large, sustained pressor responses *in vivo*, although ATZ, which elicited the greatest pressor response *in vivo*, is the weakest of these four compounds at inhibiting ecNOS *in vitro*.

S-ethyl-MEG (No. 21) caused biphasic increases in MAP (Figure 5): MAP increased sharply immediately following the injection, but returned within 2 min to a level which was still above basal MAP. (These large transient increases in MAP are not represented in Figure 5, which shows MAP at 5 min after the injection.) These pressor effects were largely attenuated by

Table 3 EC_{50} values of AATUs, MAGs and other compounds for their inhibition of the activities of iNOS, ecNOS, bNOS in tissue homogenates

No.	Compound	iNOS	EC_{50} (μ M) ecNOS	bNOS
1	N ^G -methyl-L-arginine (L-NMA)	17	5	20
2	N ^G -nitro-L-arginine (L-NOARG)	300	2	0.8
2	Aminoguanidine	80	2600	220
4	Aminoethyl-TU (AETU)	1.7	50	27
5	Aminopropyl-TU (APTU)	0.9	5.5	3
14	N-methyl-APTU	7	3	15
18	Mercaptoethylguanidine (MEG)	11.5	110	60
19	Mercaptopropylguanidine (MPG)	7	4	80
20	S-methyl-MEG	1.4	43	8
21	S-ethyl-MEG	30	850	460
22	2-Aminothiazoline	0.8	12	4
23	Guanidylethylenedisulphide (GED)	110	630	180

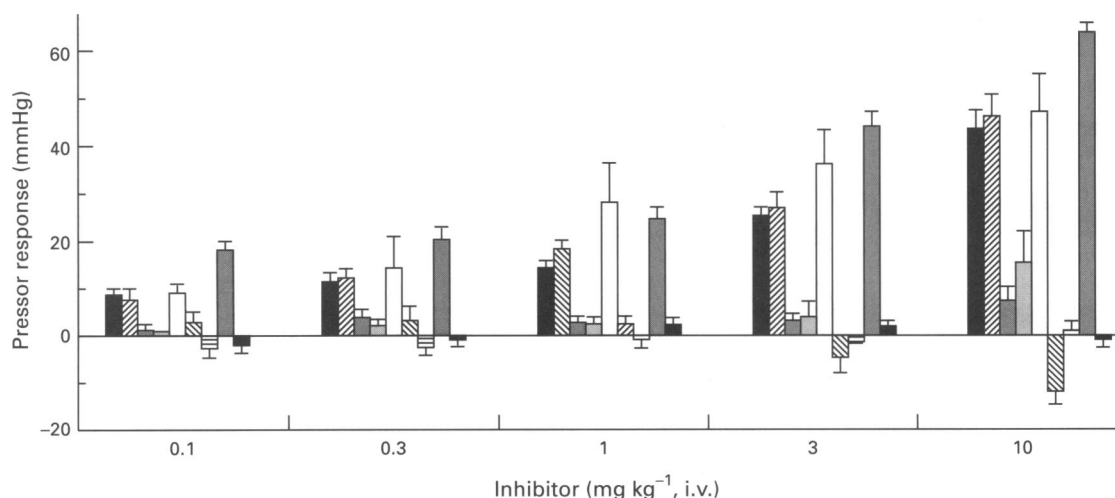


Figure 4 Changes in MAP of normal anaesthetized rats 5 min after bolus injections at doses indicated of (left to right): L-NMA (solid columns), L-NOARG (broad diagonally hatched columns), S-methyl-MEG (dark grey columns), S-ethyl-MEG (light grey columns), MPG (open columns), MEG (diagonally hatched columns), AETU (horizontally hatched columns), ATZ (dark grey columns) and GED (black columns). Baseline MAP varied between 107 ± 5 and 115 ± 5 in the various groups of rats (mean $\pm$ s.e.mean) and was not significantly different between groups ($n = 4-6$).

a bolus of L-arginine (300 mg kg^{-1}) given 10 min prior to the inhibitors (not shown). S-methyl-MEG (No. 20) produced only slight increases in MAP, despite the fact that it appears to be more potent than S-ethyl-MEG on eNOS. These were preceded by a transitory ($<1 \text{ min}$) reduction in MAP (Figure 5). Bolus injections of GED (No. 23), the disulphide of MEG, to normal rats decreased their MAP by as much as 60 mmHg (Figure 5). MAP returned to normal after 2–3 min and so these large transient decreases are not represented in Figure 5.

The changes in MAP in response to AETU were also phasic. At doses up to 10 mg kg^{-1} , initial increases in MAP (Figures 5–6) of less than 1 min duration were observed, which declined to basal values. These increases were generally slight (less than 10 mmHg at doses up to 10 mg kg^{-1}), but at doses of $30\text{--}60 \text{ mg kg}^{-1}$ they became more pronounced (Figure 6). When L-arginine (300 mg kg^{-1} i.v.) was given before AETU, the increase in MAP was attenuated (not shown). When L-NAME, a potent inhibitor of eNOS, was given prior to AETU, AETU caused no further increase in MAP, and at higher doses (60 mg kg^{-1}), small depressor effects were seen (Figure 6). L-NAME (10 mg kg^{-1} i.v.) was still able to increase MAP when injected 5 min after AETU (60 mg kg^{-1}) (Figure 6).

MEG (No. 18), on the other hand, did not cause noticeable increases in MAP at doses up to 60 mg kg^{-1} . There was, however, a transient fall in MAP immediately after injection of the drug (Figures 5–6), which somewhat resembled the fall seen with GED (Figure 5) and was not inhibited by L-NAME pretreatment (Figure 6). When L-NAME (10 mg kg^{-1} bolus, i.v.) was given following a high dose of MEG (e.g. 60 mg kg^{-1}), there was an increase in MAP (Figure 6). Thus, the pressor effect of L-NAME is not masked by MEG, even at high doses.

Infusions of AETU and MEG into normal rats produced modest net falls in MAP over 90 min, while infusions of the potent eNOS inhibitor ATZ (No. 22) markedly increased

MAP (Figures 7, 8). In rats previously treated with LPS (15 mg kg^{-1} , i.v.), infusion of AETU or MEG (starting from 90 min after the LPS injection) raised MAP by approximately 20 mmHg over 90 min, thereby restoring around 80% of the LPS induced hypotension (Figures 7, 8). A similar infusion of ATZ (No. 22) into LPS-treated rats caused an increase in MAP of 40 mmHg, an additional 20 mmHg over its effect in control rats (Figure 8). Infusion of MEG, AETU and ATZ into LPS-treated rats caused a significant reduction in iNOS activity in lung homogenates *ex vivo* by 59 ± 3 , 54 ± 2 and $83 \pm 1\%$, respectively (mean $\pm$ s.e.mean, $P < 0.05$, $n = 3\text{--}4$).

Effects of AETU, ATZ and MEG on the isometric contraction of aortic rings

AETU (No. 4), MEG (No. 18) and ATZ (No. 22) showed marked differences in their effects on precontracted rings of rat aortae (Figure 9). ATZ significantly inhibited the relaxant re-

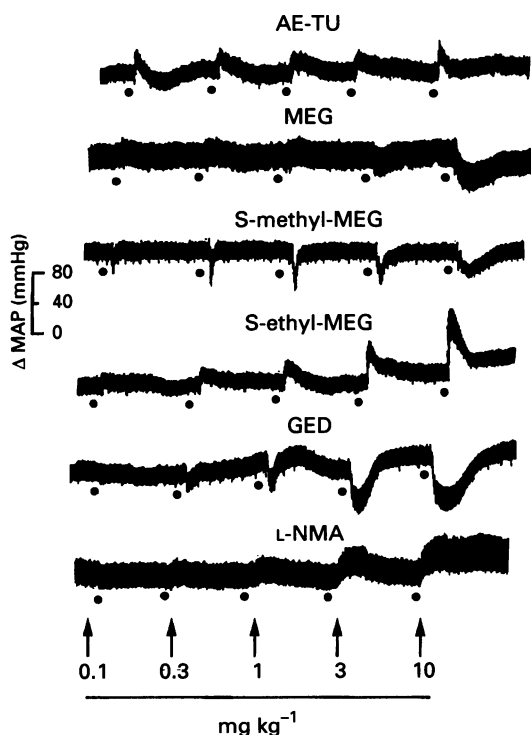


Figure 5 Representative tracings of MAP of normal anaesthetized rats given bolus injections of AETUs or MEGs ($0.1\text{--}10 \text{ mg kg}^{-1}$, i.v.). Baseline MAP varied between 102 and 120 mmHg in the individual animals depicted in these figures: (●) represent the times of injection (increasing, cumulative doses of 0.1, 0.3, 1, 3 and 10 mg kg^{-1} , i.v.). The injections were 5 min apart.

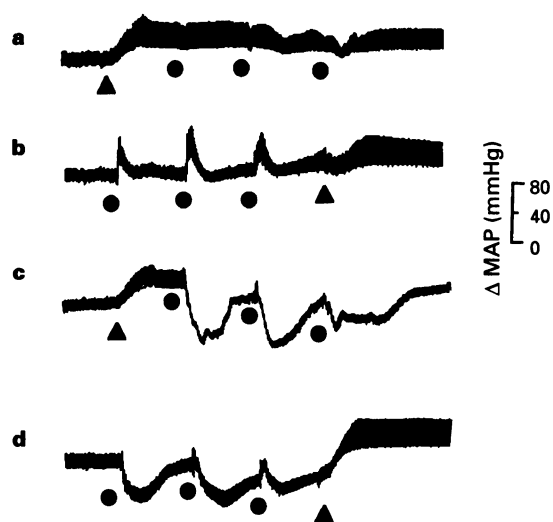


Figure 6 Representative tracings showing the effect of bolus injections of L-NAME (10 mg kg^{-1} , i.v.) on the MAP of anaesthetized rats when given prior to (a) or after (b) injection of AETU (10 , 30 and 60 mg kg^{-1}) or prior to (c) or after (d) injection of MEG. Baseline MAP varied between 123 and 132 mmHg in the individual animals depicted in these figures. (▲) represent the time of L-NAME injection (10 mg kg^{-1} , i.v.), (●) represent the times of the injection of AETU or MEG (increasing, cumulative doses of 10 , 30 and 60 mg kg^{-1} , i.v.). The injections were 5 min apart.

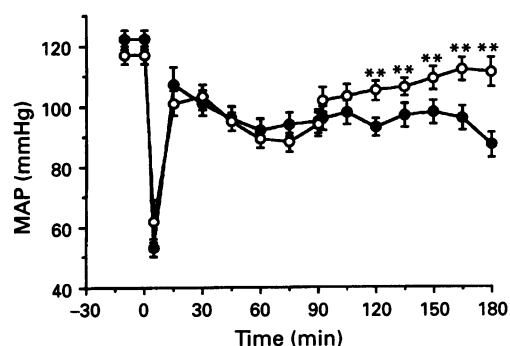


Figure 7 MAP of rats treated with LPS (at time 0) and subsequently injected (at 90 min) with saline (●) or AETU (10 mg kg^{-1} bolus and $30 \text{ mg kg}^{-1} \text{ h}^{-1}$ infusion, ○). Data are expressed as mean $\pm$ s.e.mean of $n = 4\text{--}6$ animals.

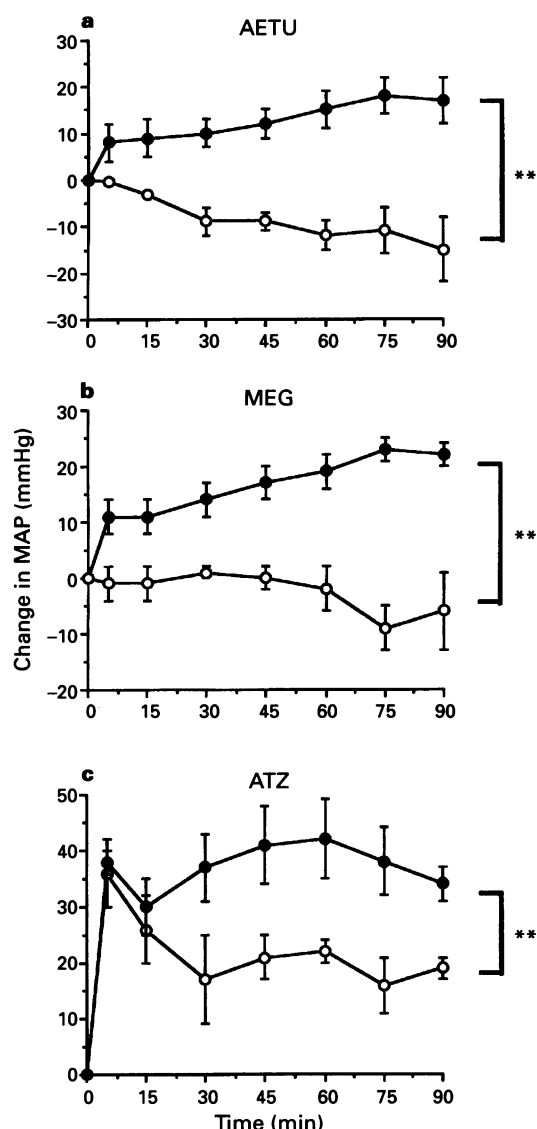


Figure 8 Relative changes in MAP in normal (○) and LPS-treated rats (●) in response to infusions of AETU, MEG or ATZ: Rats were injected with AET, MEG or ATZ (10 mg kg^{-1} , i.v.) and then the drug infused ($30 \text{ mg kg}^{-1} \text{ h}^{-1}$ in 0.2 ml kg^{-1}) for 90 min. In the LPS-treated rats, LPS (15 mg kg^{-1} , i.v.) was injected at time 0 and the drugs were administered from 90 min after LPS. Data are expressed as mean $\pm$ s.e. mean of $n=4-6$ animals.

sponse to acetylcholine at $10 \mu\text{M}$ and caused an almost complete abolition of the relaxations at $100 \mu\text{M}$. On the other hand, MEG caused no significant attenuation of relaxation, but at a concentration of $100 \mu\text{M}$, it caused an enhancement of the relaxation to lower doses of acetylcholine. The effects of AETU were intermediate between the other two compounds (Figure 9). GED ($10-100 \mu\text{M}$) caused endothelium-independent relaxation of aortic rings taken from normal rats ($n=3-4$, not shown).

In aortic rings taken from rats previously treated with LPS, AETU, MEG and ATZ ($100 \mu\text{M}$) partially reversed the hyporeactivity of the tissue to noradrenaline to a similar extent (Figure 10).

Discussion

Guanidines, isothioureas and amidines have all been reported as inhibitors of NOS, many with potencies greater than that of L-NMA. Two such isothioureas, AETU and APTU, ap-

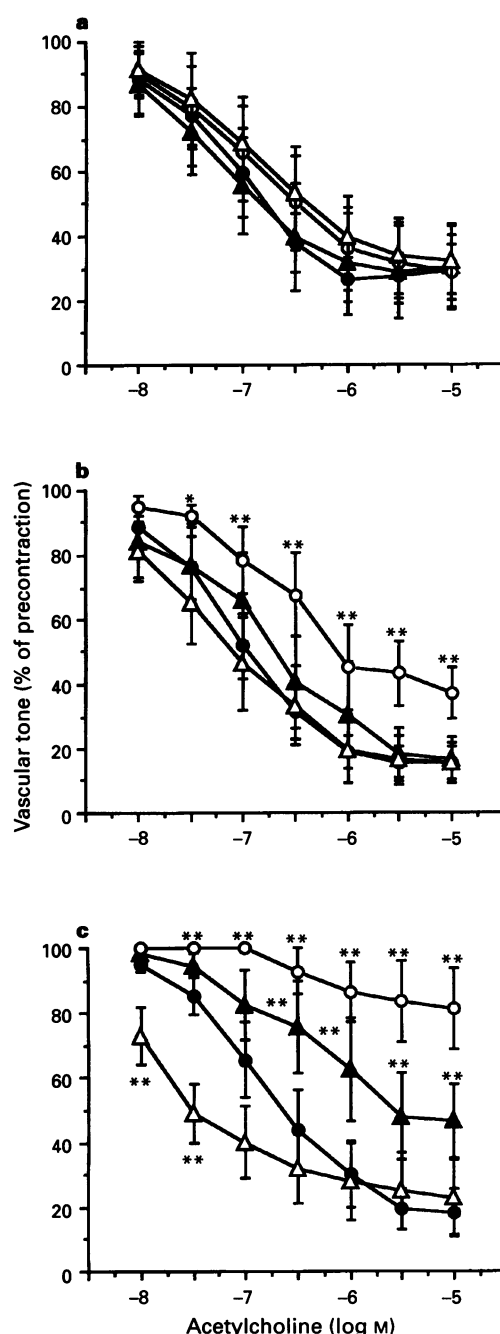


Figure 9 Concentration-response curves for acetylcholine in intact aortic rings precontracted with noradrenaline (10^{-7} M) (●) and in aortic rings pretreated with AETU (▲), MEG (△) and ATZ (○); each of the inhibitors at concentrations of $1 \mu\text{M}$ (a); $10 \mu\text{M}$ (b) and $100 \mu\text{M}$ (c). Data shown represent mean $\pm$ s.e. mean of data obtained from $n=5-7$ rings.

pear to be very potent indeed, with AETU showing a preference towards iNOS when studied in rodent and bovine eNOS and iNOS enzymes (Southan *et al.*, 1995a). We, therefore, investigated the properties of these and related compounds in solution and in pharmacological models of NOS activity.

Rearrangement of AATUs in solution

It became evident that some AATUs exhibit a chemistry in solution different from that of other isothioureas. When aqueous solutions of compounds Nos. 4, 5, 9-17 (Table 1) are brought to pH 7 or above, different chemical species are produced as indicated by (1) isolation of picrate and flavinate salts

with melting points markedly different from those obtained from acid solution, (2) the disappearance of AATUs as measured by h.p.l.c. and (3) the corresponding appearance of free thiols. These same compounds all have a chain length of 2 or 3 carbon atoms between the sulphur and the amine group. The most likely explanation of these observations is the rearrangement of AATUs to mercaptoalkylguanidines (MAGs) as outlined in Figure 11 (see also: Doherty *et al.*, 1957; Khym *et al.*, 1957).

Addition of picric acid (or flavionic acid) to solutions of AATU salts in water causes precipitation of the insoluble pi-

crate (or flavionate) of AATU. In neutral or basic solution, liberation of the free amine ($-\text{NH}_2$) allows nucleophilic attack of the amidine (central) carbon. This may result in a cyclic intermediate or transition state which can then give the MAG, bearing a free thiol group and the guanidine function. Addition of picric acid now causes the precipitation of MAG picrate. In this model, rearrangement of aminobutyl-TU (No. 6) would involve an unfavourable seven-membered intermediate and the rearrangement of S-(dimethylaminoethyl)-TU (No. 7) and S-(dimethylaminopropyl)-TU (No. 8) is prohibited by the tertiary amine group (on R in Figure 1). Thus, these three compounds do not rearrange appreciably under these conditions.

Substitution of the amidine nitrogens does not prevent rearrangement (Nos. 9–17), but it does alter the rate of the reaction and yield of MAG (Figure 3) (and the potency of NOS inhibition). The small methyl or ethyl substituents should have little steric influence, but may reduce the susceptibility of the amidine carbon to nucleophilic attack by the amine group. The yields of less than 100% thiol per AATU, even after 24 h, together with the inability to detect AATUs by h.p.l.c. after a few hours, suggest that species other than MAGs are formed from AATUs. These may include cyclic compounds as illustrated in Figure 11 or the disulphide dimers of MAGs, e.g. GED, from the oxidation of MEG.

Comparison of the effects of AATUs and their isomeric MAGs

The decomposition of AATUs to species other than the corresponding MAG probably accounts for some of the differences found when comparing the effects of AETU (No. 4) and APTU (No. 5) on NOS activity to those of MEG (No. 18) and MPG (No. 19). While the EC_{50} values for AETU ($14 \mu\text{M}$) and MEG ($13 \mu\text{M}$) against iNOS in intact macrophages are very similar (Figure 1), AETU is consistently more potent than MEG against iNOS activity in lung homogenates. This may be explained by the formation of ATZ (No. 22) from AETU, as ATZ is much more potent than MEG in lung homogenates, but much weaker in intact cells (see Tables 1, 3). Formation of a few percent of ATZ from AETU would, therefore, have little

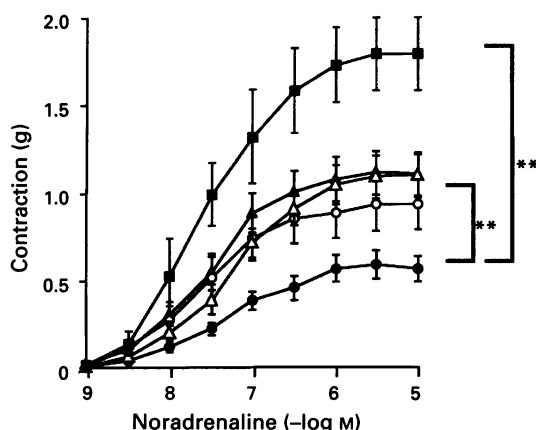


Figure 10 Concentration-response curves for noradrenaline (10^{-9} – 10^{-5} M) in endothelium-denuded aortic rings taken from rats treated with LPS (15 mg kg^{-1} , i.v. for 180 min) and vehicle (●), and the effect of *in vivo* treatment with AETU, (▲); MEG, (△); or ATZ, (○) ($100 \mu\text{M}$ for 30 min for all NOS inhibitors). Drugs (10 mg kg^{-1} bolus and $30 \text{ mg kg}^{-1} \text{ h}^{-1}$ infusion) were given from 90 min after LPS injection. Also shown are responses for rings from control rats (no LPS, ■). Data shown represent mean $\pm$ s.e. mean of data obtained from $n=5-7$ rings.

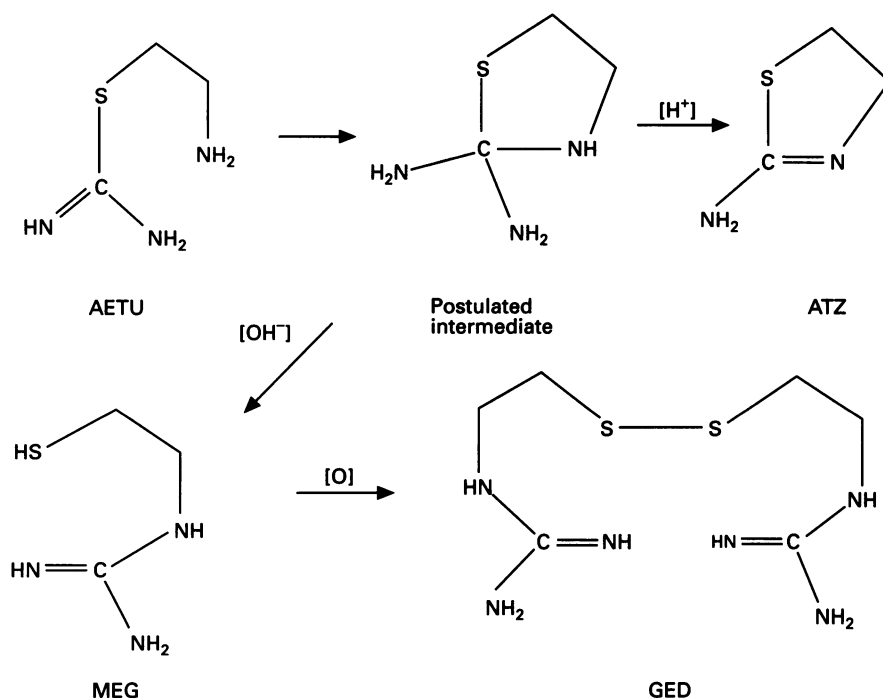


Figure 11 Scheme demonstrating the rearrangement of aminoethyl-isothioureia (AETU) to aminothiazoline (ATZ) and mercaptoethylguanidine (MEG) and the subsequent dimerization of MEG to guanidinoethyldisulphide (GED).

discernible effect on iNOS activity in macrophages when compared to MEG, but, being 15 times more potent than MEG against iNOS activity in homogenized lungs, this would give a significant augmentation of the apparent potency of AETU. Similarly, ATZ is far more potent than MEG against ecNOS and bNOS activities in homogenates. Consequently, its formation from AETU, even in small amounts, would again give apparent EC₅₀ values for AETU lower than those of MEG. In addition, the route of decomposition of AETU is sensitive to the incubation conditions: in hydrogen carbonate buffer (pH 7), for instance, ATZ is the predominant product formed from AETU (Khym *et al.*, 1958). Similar arguments can be used to explain the differences between other AATUs and their corresponding MAGs: APTU is more potent than MPG on both models of iNOS activity, despite yields of MPG in phosphate buffer of 96%. It is conceivable that other more potent products, perhaps the analogous cyclic derivative, penthiaziline (Khym *et al.*, 1958), may form from APTU.

Similarly, N-methyl-APTU (No. 14), N,N-di-methyl-APTU (No. 16) and N,N-di-methyl-AETU (No. 12) are more effective inhibitors of nitrite production by macrophages when prepared in water rather than sodium hydroxide solution (used to facilitate conversion to MAGs). In these cases, the slower rate of rearrangement may allow competing reaction to occur in the cell/organ homogenates (hence lower yield of MEG), generating other, perhaps more potent products. It is also likely that the 20 min incubation at pH ~8, used to generate MAGs from AATUs in these comparisons, leads to enhanced formation of the disulphides (Shapiro *et al.*, 1963), which are inert to the Aldrithiol-2 assay.

From the above it may be assumed that aqueous solutions of AATUs with a 2–3 carbon side chain may generate other species at physiological pH. In phosphate buffer (Figure 3) and other simple buffers these solutions should essentially be of MEG and MPG, respectively. This has been established for both AETU (Khym *et al.*, 1957) and APTU (Khym *et al.*, 1958) and appears to be so for compounds Nos. 9–12 and No. 17, although variable amounts of the disulphide dimers (GED in the case of MEG) may be formed due to aerial oxidation (Khym *et al.*, 1957; 1958). In simple neutral solutions, AATUs give predominantly MAGs, but in physiological buffers the presence of many other species/equilibria influence the decomposition of AATUs. Furthermore, in oxygenated solution, the formation of disulphides would be facilitated. These compounds may have actions as inhibitor of NOS (such as GED, Tables 1, 3) or as vasodilators (also GED). *In vivo*, AETU, MEG, GED and ATZ have been isolated from animals given AETU, but only MEG and GED are found if MEG is administered (Shapiro *et al.*, 1963; Kozak & Arient, 1973). In addition, further oxidation of GED and MEG occurs *in vivo* with the increase in oxidation state of the sulphur (Shapiro *et al.*, 1963). Similarly, taurocyanamide (compound No. 18, Table 1, but with R = –CH₂CH₂SO₃H) may be formed from MEG *in vivo*. Taurocyanamide does not, however, inhibit nitrite formation by immunostimulated macrophages (unpublished observation). Interestingly, MEG is formed following *in vivo* administration of GED (Shapiro *et al.*, 1963).

Structure-activity considerations

Although for compounds Nos. 4, 5, 9–12 and No. 17 the near-quantitative conversion to MAGs may allow some generalization about structure-activity relationships, these must be tempered with the uncertainty about (1) the extent of conversion to MAGs in physiological situations (especially *in vivo*), (2) differential cellular uptake and (3) the potential for side products with extreme potencies, such as ATZ (No. 22, EC₅₀ 0.8 µM against iNOS in the homogenates of lungs) and GED (No. 23, EC₅₀ 6 µM against nitrite production) which derive from AETU. Hence, although we could compare the reduced potencies of those AATUs with substituted amidino nitrogens to those of similarly substituted isothioureas and amidines (Southan *et al.*, 1995b), it should be noted that this difference

in potency is small for the mono-substituted derivatives of AETU and APTU (Tables 1, 3). In addition, there is a general correlation between yield, rate of generation of thiol and potency against iNOS. Moreover, the rates of oxidation of AATUs to their disulphides in medium/cells and their activities are not known.

The question arises as to whether the altered potency of the alkyl-substituted AATUs is due to the reduced intrinsic potency of the newly formed N-substituted MAG or to a reduced rate of formation and/or yield of the MAG. Over the 20 min of the assay used to measure NOS activity in tissue homogenates, slow conversion of AATUs to MAGs may result in an underestimation of inhibitory potency if MAGs (or other species) are the active principle. Synthesis and testing of the MAGs directly would answer some of the points raised for those N-alkylated compounds where MAGs cannot be generated quantitatively by neutralization of solutions of the appropriate AATU (Nos. 13–16). MEG sulphate was synthesized by a route independent of AETU and found to have EC₅₀ values against iNOS (12 µM) and ecNOS (115 µM) similar to those of solutions of MEG prepared from neutralizing solutions of AETU.

Uptake of inhibitors by macrophages

Substitution of the sulphur of MEG (No. 18) reduces the potency in inhibiting nitrite accumulation in whole cells, as exemplified by S-methyl-MEG (No. 20) and S-ethyl-MEG (No. 21). However, when tested directly against iNOS activity, S-methyl-MEG is as potent as MEG, and S-ethyl-MEG is as effective as L-NMA. This raises questions about the uptake of MAGs by intact cells and the role of the free thiol group in this respect. MAGs that contain a free thiol (those derived from the appropriate AATUs) possess similar potencies in both intact cells and lung homogenates. The disulphide, GED (No. 23), however, is more potent than its 'monomer' MEG against iNOS in whole cells (macrophages), but is 100 times weaker against iNOS activity in homogenates. The hypothesis that GED may actively be taken up by the cells and perhaps reduced to the active MEG intracellularly needs further exploration. Interestingly, GED has adverse effects on cell respiration at concentrations of 100 µM–1 mM, which could conceivably be brought about by changes in the redox status of the cell.

There is an approximate 100 fold difference between the efficacies of ATZ against iNOS activity in homogenized lungs and against nitrite production by intact cells, indicating, as others have found (Garvey *et al.*, 1994), that the utility of some isothioureas as inhibitors of iNOS may be limited by poor cellular uptake.

Mechanism of action by MAGs

Inhibition of nitrite formation by induced macrophages by AATUs and MEGs is attenuated with increasing concentrations of L-arginine (Table 2). This suggests that these compounds may compete with L-arginine for its binding site on NOS. This effect was also noted with the inhibition of iNOS activity in homogenates of rat lung (unpublished data). The nature of the inhibition has yet to be fully investigated, but preliminary results in purified iNOS and ecNOS suggest that the inhibition of iNOS by MEG is competitive against L-arginine (D. Wolff, R. Bryk, G.J. Southan, C. Szabo, unpublished observations). This is in contrast to the effects of aminoguanidine which shows characteristics of a progressive inhibition of iNOS (Joly *et al.*, 1994; Wu *et al.*, 1995; Wolff *et al.*, 1995). By analogy with other guanidines, it is likely that MAGs bind to the haeme of NOS either through the guanidine group or, possibly, the sulphur. As with other inhibitors that contain sulphur or selenium, there is always the possibility of interaction with cysteine residues in the vicinity of the catalytic site.

Certain NOS inhibitors, for example L-NMA, inhibit the uptake of L-arginine in macrophages, thus reducing substrate

availability for iNOS (Bogle *et al.*, 1992; Baydoun *et al.*, 1994). Such effects may also contribute to the inhibitory actions of some MAGs on NO production by macrophages.

Selectivity of mercaptoalkylguanidines for iNOS

MEG and S-methyl-MEG, while relatively potent against iNOS activity in homogenates of lung, are weak inhibitors of ecNOS activity in homogenates of endothelial cells from bovine aortae and cause relatively modest pressor responses *in vivo* (Figure 6). S-ethyl-MEG shows similar selectivity for iNOS inasmuch as it is weaker than L-NMA against ecNOS, while being far more potent than L-NMA against iNOS. Most MAGs are also weaker against NOS activity in the brain homogenates (bNOS), although MPG and its N-methyl derivative appear to show little discrimination between constitutive and inducible isoforms.

The indications of potential selectivity of some of these agents are reinforced by the *in vivo* data where responses of MAP to doses of MAGs were recorded. Changes of MAP in response to administration of drugs are a reflection of their effects on ecNOS and, on the whole, there is reasonable agreement between the *in vitro* data on ecNOS and the *in vivo* data (pressor responses): AETU and MEG are weak against ecNOS activity and exert only weak pressor effects *in vivo*.

The lack of increase in MAP when MEG was injected at doses up to 10 mg kg⁻¹ is encouraging in terms of *in vivo* selectivity towards iNOS. Depressor effects (due to direct vasodilator action) at these high doses are unlikely to be masking pressor effects, as these depressor effects are unaltered by pretreatment with L-NAME. These findings also imply that the depressor effects are not related to NOS, a finding in agreement with their insensitivity to L-arginine. Similar depressor effects of guanidines and amidines have been observed previously (Fastier & Smirk, 1947; Fastier, 1962; Ozawa & Sugawara, 1968). It is interesting in this respect that we found GED to be a potent endothelium-independent vasodilator. The mechanism of action remains to be elucidated, but the formation of GED from AETU and/or MEG *in vivo* (see above) may explain the vasodilator actions of these compounds.

In vivo effects of AATUs and MAGs

As mentioned above, there is reasonable agreement between the *in vitro* data on ecNOS and the *in vivo* data. MPG and ATZ are as potent as L-NMA and L-NOARG against ecNOS *in vitro*, and all give large increases in MAP in anaesthetized rats. On the other hand, AETU and MEG, both weak inhibitors of ecNOS activity *in vitro*, did not cause significant, sustained increases in MAP in normal rats (Figure 5). However, certain discrepancies were also noted, such as the difference between the potency of S-methyl and S-ethyl-MEG on ecNOS *in vitro* vs. the difference in their pressor responses (S-methyl-MEG is more potent on ecNOS *in vitro* but is a less potent pressor agent), which needs further clarification.

Although the sustained increases in MAP due to AETU in normal rats were not significant, AETU gave initial increases in MAP of 1–2 min duration, which then returned to initial levels. The reason for this biphasic response to AETU is unclear, but it is tempting to speculate that (a) the initial pressor effect seen with AETU is a direct effect of AETU *in vivo* before its metabolism or (b) rapid and transient formation of ATZ is responsible for the initial transient pressor responses. It is noteworthy in this context that no biphasic responses were seen for L-NMA or ATZ. MEG, on the other hand, did not produce increases in MAP but caused transient falls in MAP of less than 1 min duration followed by total or partial recovery.

Infusions of AETU or MEG over 90 min caused increases in MAP only in rats which had previously received LPS, restoring around 80% of the LPS-induced hypotension. These increases of approximately 20 mmHg are equal to the difference between the changes induced in LPS treated rats

(40 mmHg) and control rats (20 mmHg) by an infusion of ATZ (Figures 8, 9). This suggests that AETU and MEG are as effective as ATZ in raising the fall in MAP due to endotoxin-induced NOS activity, but do not inhibit ecNOS *in vivo*. An additional indication of the potential beneficial effects of MEG/AETU against iNOS is the reduction of iNOS activity in homogenates of lungs taken from LPS treated rats that had also received an infusion of one of the above drugs 90 min after administration of LPS. A further example is the partial reversal, by all three compounds (100 µM), of the hyporeactivity to noradrenaline seen in aortic rings taken from rats previously treated with LPS. This hyporeactivity to noradrenaline is a consequence of NO production by iNOS (see: Szabó, 1995 for review). The reversal is not complete, but equivalent to that seen with millimolar doses of L-NMA, L-NAME or aminoguanidine in this particular model of *ex vivo* vascular hyporeactivity (Wu *et al.*, 1994; 1995).

The comparatively weaker effects of AETU, but particularly of MEG, on ecNOS is exemplified by the differences in the effects of AETU, MEG and ATZ on precontracted rings of rat aortae. ATZ (10 µM) caused a significant reduction in the relaxation in response to acetylcholine in line with its potent effects on ecNOS, but MEG did not interfere in the relaxant responses, other than to augment the relaxation elicited by low doses of acetylcholine. This augmentation may be due to a weak vasorelaxant response of MEG itself or by its oxidation to GED which is probably facilitated by the oxygenated Krebs buffer used in these experiments. The intermediate effects of AETU are, again, consistent with the formation of some ATZ from AETU in the carbonated Krebs buffer used in these experiments.

Conclusions and applications

Our data indicate that MAGs represent a novel class of NOS inhibitors. Some members of the class are potent inhibitors of iNOS, but are weaker on the constitutive NOS isoforms (ecNOS and bNOS). These compounds, such as MEG, S-methyl-MEG and S-ethyl-MEG, may represent examples of compounds that could be used *in vivo* for selective inhibition of iNOS. Moreover, the demonstration of the selectivity of these compounds may open new ways for the rational design of iNOS selective inhibitors of NOS.

These compounds add to the growing list of non-amino acid compounds with selectivity towards iNOS (aminoguanidine, certain isothioureas and guanidines) and support the view that when designing NOS inhibitors with selectivity for iNOS, it seems unnecessary to incorporate the amino acid group (as in analogues of L-arginine) that seems to be equally well recognised by all isoforms of NOS and imparts no selectivity towards iNOS. Thus, the study of non amino acid based compounds may well be a promising approach to designing selective inhibitors of iNOS (see also: Southan & Szabó, 1996).

The formation of MAGs from AATUs occurs *in vitro* and *in vivo*, and may be responsible for the biological actions of AATUs, as exemplified by AETU and MEG. Thus, the beneficial effects of AETU on iNOS-dependent pathophysiological processes in endotoxaemia, such as hypotension, liver damage (Thiemermann *et al.*, 1995) and endotoxin-induced lethality (Szabó *et al.*, 1995), are likely to be due to its metabolite, MEG. In view of the *in vivo* generation of ATZ from AETU (see above) and the lack of effects of MEG on MAP, the use of MAGs would be preferable to using AATUs for the selective inhibition of iNOS. However, the oxidation of MAGs to the corresponding disulphides (such as GED), which may have potent depressor and vasodilator effects, may hamper the delineation of the actions of MAGs and related compounds. It may, therefore, be worthwhile developing further examples of S-substituted MAGs as these are not susceptible to oxidation of the thiol group.

MAGs and their related compounds are, clearly, an interesting, heterogeneous group of compounds that include ex-

amples of potent inhibitors of NOS, selective inhibitors of iNOS and potent (endothelium-independent) vasodilators. The mechanisms of many of these diverse actions require further investigation. Nevertheless, MAGs may be of use in the experimental therapy of pathophysiological conditions associated with NO overproduction by iNOS. It is also worthy of note that mercaptoalkylguanidines, including MEG, have been described as a radioprotective agents (Shapiro *et al.*, 1957; Hino *et al.*, 1966; Kozak & Arient, 1973) in view of the recent report that L-arginine based inhibitors of NOS *in vivo* also protect against exposure to radiation (Liebmann *et al.*, 1994).

References

- BAYDOUN, A.R., BOGLE, R.G., PEARSON, J.D. & MANN, G.E. (1994). Discrimination between citrulline and arginine transport in activated murine macrophages: inefficient synthesis of NO from recycling of citrulline to arginine. *Br. J. Pharmacol.*, **112**, 487–492.
- BOGLE, R.G., MONCADA, S., PEARSON, J.D. & MANN, G.E. (1992). Identification of inhibitors of nitric oxide synthase that do not interact with the endothelial cell L-arginine transporter. *Br. J. Pharmacol.*, **105**, 768–770.
- CORBETT, A., TILTON, R.G. & CHANG, K. (1992). Aminoguanidine, a novel inhibitor of nitric oxide formation, prevents diabetic vascular dysfunction. *Diabetes*, **41**, 552–556.
- DAWSON, V.M., DAWSON, T.M., LONDON, E.D., BRETT, D.S. & SNYDER, S.H. (1991). Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 6368–6371.
- DOHERTY, D.G., SHAPIRA, R. & BURNETT, W.T. (1957). Synthesis of aminoalkylisothiuronium salts and their conversion to mercaptoalkylguanidines and thiazolines. *J. Am. Chem. Soc.*, **79**, 5667–5671.
- DINERMAN, J.L., LOWENSTEIN, C.J. & SNYDER, S.H. (1993). Molecular mechanisms of nitric oxide production. Potential relevance to cardiovascular disease. *Circ. Res.*, **73**, 217–222.
- FASTIER, F.N. (1962). Structure activity relationships of amidine derivatives. *Pharmacol. Rev.*, **14**, 37–90.
- FASTIER, F.N. & SMIRK, F. (1947). Circulatory properties of isothioureas, guanidines, iso-ureas and amidines. *J. Pharmacol.*, **89**, 256–270.
- GARTHWAITE, J. (1991). Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neurosci.*, **14**, 60–67.
- GARVEY, E.P., OPLINGER, J.A., TANOURY, G.J., SHERMAN, P.A., FOWLER, M., MARSHALL, S., HARMON, M.F., PAITH, J.E. & FURFINE, E.S. (1994). Potent and selective inhibition of human nitric oxide synthases. *J. Biol. Chem.*, **269**, 26669–26676.
- GRASETTI, D.R. & MURRAY, J.F. (1967). Determination of sulphydryl groups with 2,2'-or 4,4'-dithiopyridine. *Arch. Biochem. Biophys.*, **119**, 41–49.
- GROSS, S.S., JAFFE, E.A., LEVI, R. & KILBOURN, R.G. (1991). Cytokine-activated endothelial cells express an isotype of nitric oxide synthase which is tetrahydrobiopterin-dependent, calmodulin-independent and inhibited by arginine analogs with a rank-order of potency characteristic of activated macrophages. *Biochem. Biophys. Res. Commun.*, **187**, 823–829.
- GROSS, S.S., STUEHR, D.J., AISAKA, K., JAFFE, E.A., LEVI, R. & GRIFFITH, O.W. (1990). Macrophage and endothelial cell nitric oxide synthesis: cell-type selective inhibition by N^G-aminoarginine, N^G-nitroarginine and N^G-methylarginine. *Biochem. Biophys. Res. Commun.*, **170**, 96–103.
- HASAN, K., HEESSEN, B.J., CORBETT, J.A., MCDANIEL, M.L., CHANG, K., ALLISON, W., WOLFENBUTTEL, B.H.R., WILLIAMSON, J.R. & TILTON, R.G. (1993). Inhibition of nitric oxide formation by guanidines. *Eur. J. Pharmacol.*, **249**, 101–106.
- HINO, T., TANA-AMI, K., YAMADA, K. & AKABOSHI, S. (1966). Radiation-protective agents. I. Studies on N-alkylated-2-(2-aminoethyl)thiopseudoureas and 1,1-(dithioethylene)diguanidines. *Chem. Pharmacol. Bull.*, **14**, 1193–1201.
- JOLY, G.A., AYRES, M., CHELLY, F. & KILBOURN, R.G. (1994). Effects of N^G-methyl-L-arginine, N^G-nitro-L-arginine and aminoguanidine on constitutive and inducible nitric oxide synthase in rat aorta. *Biochem. Biophys. Res. Commun.*, **199**, 147–154.
- KHYM, J.X., DOHERTY, D.G. & SHAPIRA, R. (1958). Ion exchange studies of tranquanylation reactions. II. Rearrangement of 3-aminopropylisothiurea and N-substituted aminoethyl- and aminopropylisothiureas to mercaptoalkylguanidines and 2-aminothiazolines or penthiazolines. *J. Am. Chem. Soc.*, **80**, 3342–3349.
- KHYM, J.X., SHAPIRA, R. & DOHERTY, D.G. (1957). Ion exchange studies of tranquanylation reactions. I. Rearrangement of S,2-aminoethylisothiurea to 2-mercaptoethylguanidine and 2-aminothiazoline. *J. Am. Chem. Soc.*, **79**, 5663–5666.
- KOZAK, I. & ARIENT, M. (1973). Metabolism and radioprotective effect of AET and MEG in organism. *Strahlentherapie*, **146**, 367–373.
- LAMBERT, L.E., WHITTEN, J.P., BARON, B.M., CHENG, H.C., DOHERTY, N.S. & MCDONALD, I.A. (1991). Nitric oxide synthesis in the CNS, endothelium and macrophages differs in its sensitivity to inhibition by arginine analogues. *Life Sci.*, **48**, 69–75.
- LIEBMAN, J., DELUCA, A.M., COFFIN, D., KEEFER, L.K., VENZON, D., WINK, D.A. & MITCHELL, J.B. (1994). In vivo radiation protection by nitric oxide modulation. *Cancer Res.*, **54**, 3365–3368.
- MACALLISTER, R.J., WHITELY, G.S. & VALLANCE, P. (1994). Effects of guanidino and uremic compounds on nitric oxide pathways. *Kidney Int.*, **45**, 737–742.
- MARLETTA, M.A. (1993). Nitric oxide synthase structure and mechanism. *J. Biol. Chem.*, **268**, 12231–12234.
- MISKO, T.P., MOORE, W.M., KASTEN, T.P., NICKOLS, D.A., CORBETT, J.A., TILTON, R.G., MCDANIEL, M.L., WILLIAMSON, J.R. & CURRIE, M.G. (1993). Selective inhibition of the inducible NO synthase by aminoguanidine. *Eur. J. Pharmacol.*, **233**, 119–125.
- NAKANE, M., KLINGHOFFER, V., KUK, J.E., DONNELLY, J.L., BUDZIK, G.P., POLLOCK, J.S., BASHA, F. & CARTER, G.W. (1995). Novel potent and selective inhibitors of inducible nitric oxide synthase. *Mol. Pharmacol.*, **47**, 831–834.
- NATHAN, C. (1992). Nitric oxide as a secretory product of mammalian cells. *FASEB J.*, **6**, 3051–3064.
- OZAWA, H. & SUGAWARA, K. (1968). Structure-activity relationship of alkylguanidines on smooth muscle organs. *Chem. Pharmacol. Bull.*, **16**, 2376–2382.
- SHAPIRO, R., DOHERTY, D.G. & BURNETT, W.T. (1957). Chemical protection against ionizing radiation. III. Mercaptoalkylguanidines and related isothiuronium compounds with protective activity. *Radiation Res.*, **7**, 22–34.
- SHAPIRO, B., SCHWARTZ, E.E. & KOLLERMANN, G. (1963). The mechanism of action of AET. *Radiation Res.*, **18**, 17–30.
- SNYDER, S. & BRETT, D.S. (1992). Biological roles of nitric oxide. *Sci. Am.*, **166**, 28–35.
- SOUTHAN, G.J. & SZABÓ, C. (1996). Commentary: Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. *Biochem. Pharmacol.* (in press).
- SOUTHAN, G.J., SZABÓ, C., O'CONNOR, M.P., SALZMAN, A.L. & THIEMERMANN, C. (1995a). Amidines are potent inhibitors of constitutive and inducible nitric oxide synthases: preferential inhibition of the inducible isoform. *Eur. J. Pharmacol.* (in press).
- SOUTHAN, G.J., SZABÓ, C. & THIEMERMANN, C. (1995b). Isothioureas: potent inhibitors of nitric oxide synthases with variable isoform selectivity. *Br. J. Pharmacol.*, **114**, 510–516.
- SZABÓ, C. (1995). Alterations in nitric oxide production in various forms of circulatory shock. *New Horizons*, **3**, 2–32.

- SZABÓ, C., SOUTHAN, G.J. & THIEMERMANN, C. (1994a). Beneficial effects and improved survival in rodent models of septic shock with S-methyl-isothiouraea sulfate, a novel, potent and selective inhibitor of inducible nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 12472–12476.
- SZABÓ, C., SOUTHAN, G.J. & THIEMERMANN, C. (1995). Non-arginine-based selective inhibitors of inducible nitric oxide synthase exert beneficial effects in endotoxic shock. *Shock*, (Suppl 1) **3**, S72.
- SZABÓ, C., SOUTHAN, G.J., THIEMERMANN, C. & VANE, J.R. (1994b). Aldehyde metabolites account for the inhibitory effect of polyamines on the induction of nitric oxide synthase. *Br. J. Pharmacol.*, **113**, 757–766.
- SZABÓ, C. & THIEMERMANN, C. (1993). Potential therapeutic use of nitric oxide synthase inhibitors. *Current Op. Develop. Drugs*, **2**, 1165–1174.
- THIEMERMANN, C., RUTTEN, H., WU, C.C. & VANE, J.R. (1995). The multiple organ dysfunction syndrome caused by endotoxin in the rat: attenuation of liver dysfunction by inhibitors of nitric oxide synthase. *Br. J. Pharmacol.*, **116**, 2845–2851.
- VANE, J.R. (1994). The Croonian lecture, 1993: The endothelium: maestro of the blood circulation. *Proc. R. Soc. B.*, **343**, 225–246.
- WOLFF, D.J. & LUBESKIE, A. (1995). Aminoguanidine is an isoform-selective, mechanism-based inactivator of nitric oxide synthase. *Arch. Biochem. Biophys.*, **316**, 290–301.
- WORRALL, N., LAZENBY, W., MISKO, T., LIN, T., RODI, C., MANNING, P., TILTON, R., WILLIAMSON, J. & FERGUSON, JR. T. (1995). Modulation of in vivo alloreactivity by inhibition of inducible nitric oxide synthase. *J. Exp. Med.*, **181**, 63–70.
- WU, C.C., CHEN, S.J., SZABÓ, C., THIEMERMANN, C. & VANE, J.R. (1995). Aminoguanidine inhibits the delayed circulatory failure in endotoxic shock in the anaesthetized rat. *Br. J. Pharmacol.*, **114**, 1666–1672.
- WU, C.C., SZABÓ, C., CHEN, S.J., THIEMERMANN, C. & VANE, J.R. (1994). Activation of soluble guanylyl cyclase by a factor other than nitric oxide or carbon monoxide contributes to the vascular hyporeactivity to vasoconstrictor agents in the aorta of rats treated with endotoxin. *Biochem. Biophys. Res. Commun.*, **201**, 436–442.
- XIE, Q. & NATHAN, C. (1994). The high-output nitric oxide pathway: role and regulation. *J. Leukoc. Biol.*, **56**, 576–582.

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