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Ruthenium complexes as nitric oxide scavengers: a potential therapeutic approach to nitric oxide-mediated diseases

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1 Ruthenium(III) reacts with nitric oxide (NO) to form stable ruthenium(II) mononitrosyls. Several Ru(III) complexes were synthesized and a study made of their ability to bind NO, *in vitro* and also in several biological systems following expression of the inducible isoform of nitric oxide synthase (iNOS). Here we report on the properties of two, related polyaminocarboxylate-ruthenium complexes: potassium chloro[hydrogen(ethylenedinitrilo)tetraacetato]ruthenate (=JM1226; CAS no.14741-19-6) and aqua[hydrogen(ethylenedinitrilo)tetraacetato]ruthenium (=JM6245; CAS no.15282-93-6).

2 Binding of authentic NO by aqueous solutions of JM1226 yielded a product with an infrared (IR) spectrum characteristic of an Ru(II)-NO adduct. A compound with a similar IR spectrum was obtained after reacting JM1226 with S-nitroso-*N*-acetylpenicillamine (SNAP).

3 The effect of JM1226 or JM6245 on nitrite (NO_2^-) accumulation in cultures of macrophages (RAW 264 line) 18 h after stimulating cells with lipolysaccharide (LPS) and interferon-γ (IFNγ) was studied. Activation of RAW264 cells increased NO_2^- levels in the growth medium from (mean $\pm$ 1 s.e.mean) 4.9 ± 0.5 μM to 20.9 ± 0.4 μM. This was blocked by actinomycin D (10 μM) or cycloheximide (5 μM). The addition of JM1226 or JM6245 (both 100 μM) to activated RAW264 cells reduced NO_2^- levels to 7.6 ± 0.2 μM and 8.8 ± 0.6 μM, respectively. N^G-methyl-L-arginine (L-NMMA; 250 μM) similarly reduced NO_2^- levels, to 6.1 ± 0.2 μM.

4 The effect of JM1226 or JM6245 on NO-mediated tumour cell killing by LPS + IFNγ-activated macrophages (RAW 264) was studied in a co-culture system, using a non-adherent murine mastocytoma (P815) line as the 'target' cell. Addition of JM1226 or JM6245 (both 100 μM) to the culture medium afforded some protection from macrophage-mediated cell killing: target cell viability increased from $54.5 \pm 3.3\%$ to $93.2 \pm 7.1\%$ and $80.0 \pm 4.6\%$, respectively ($n=6$).

5 Vasodilator responses of isolated, perfused, pre-contracted rat tail arteries elicited by bolus injections (10 μl) of SNAP were attenuated by the addition of JM1226 or JM6245 (10^{-4} M) to the perfusate: the ED_{50} increased from 6.0 μM (Krebs only) to 1.8 mM (Krebs + JM6245) and from 7 μM (Krebs only) to 132 μM (Krebs + JM1226). Oxyhaemoglobin (5 μM) increased the ED_{50} value for SNAP from 8 μM to 200 μM.

6 Male Wistar rats were injected with bacterial LPS (4 mg kg^{-1}; i.p.) to induce endotoxaemia. JM1226 and JM6245 (both 100 μM) fully reversed the hyporesponsiveness to phenylephrine of tail arteries isolated from animals previously (24 h earlier) injected with LPS. Blood pressure recordings were made in conscious LPS-treated rats using a tail cuff apparatus. A single injection of JM1226 (100 mg kg^{-1}, i.p.) administered 20 h after LPS (4 mg kg^{-1}, i.p.) reversed the hypotension associated with endotoxaemia.

7 The results show that JM1226 and JM6245 are able to scavenge NO in biological systems and suggest a role for these compounds in novel therapeutic strategies aimed at alleviating NO-mediated disease states.

Keywords: Nitric oxide scavengers; ruthenium(III) polyaminocarboxylates; endotoxaemia; hypotension; vascular hyporeactivity; nitric oxide synthase

Introduction

The inducible isoform of nitric oxide synthase (type II NOS or iNOS) is expressed in a variety of cells in response to stimulation by bacterial lipopolysaccharide (LPS) and/or certain inflammatory cytokines, such as tumour necrosis factor α (TNFα), interferon γ (IFNγ) and interleukins (IL) 1 and 2 (Iyengar *et al*., 1987). Once expressed, iNOS is maximally active and remains so for several hours, generating high levels of NO. Transcriptional activation of this 'high-output' pathway for NO has been implicated in the pathogenesis of several important disease states, most notably in septic shock (Thiemermann & Vane, 1990; Stoclet *et al*., 1993; Szabo *et al*., 1993). This is a life-threatening condition caused by a severe bacterial infection, characterised by peripheral vasodilation and profound hypotension (>40 mmHg fall in systolic pressure), compounded by a greatly diminished vascular reactivity to vasoconstrictor agents (Glauser *et al*., 1991). Septic shock results in poor tissue perfusion which can lead to multiple organ failure and ultimately death.

Evidence that iNOS-generated NO is an important causal factor in the septic shock syndrome comes from several sources. First, iNOS expression has been detected directly in several tissues (heart, lung, liver, spleen, kidney, brain and skeletal muscle) within a few hours after treating rats with LPS (Knowles *et al*., 1990; Szabo *et al*., 1993; Liu *et al*., 1993; Buttery *et al*., 1994; Hom *et al*., 1995). Second, the NOS inhibitors N^G-methyl-L-arginine (L-NMMA) and N^ω-nitro-L-arginine methyl ester (L-NAME; Rees *et al*., 1990) reverse the

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hyporeactivity to catecholamines of vessels isolated from LPS-treated rats (Julou-Schaeffer *et al*., 1990; Schott *et al*., 1993). Similarly, *in vivo* studies have shown that the diminished pressor response to vasoconstrictors in endotoxic shock is reversed by NOS inhibitors (Gray *et al*., 1991; Szabo *et al*., 1993). Moreover, L-NMMA can alleviate the severe hypotension associated with endotoxaemia, both in animal models (Kilbourn *et al*., 1990; Thiemermann & Vane, 1990) and in humans (Lin *et al*., 1994; Petros *et al*., 1994). Third, and most convincingly, transgenic mice possessing a null mutation for the iNOS gene do not become hypotensive, nor do they succumb as readily as wild-type animals to treatment with bacterial endotoxin (MacMicking *et al*., 1995; Wei *et al*., 1995).

The ability of NOS inhibitors to alleviate some of the haemodynamic manifestations of septic shock has focused attention on their therapeutic potential. The outcome thus far has been equivocal as adverse cardiovascular consequences have been observed, for example aggravated pulmonary hypertension (Robertson *et al*., 1994), together with reports of increased mortality in animal models (Minnard *et al*., 1994; Tracey *et al*., 1995). The capacity to discriminate between iNOS and constitutive isoforms of NOS (cNOS) is important in this context and the search for inhibitors with greater selectivity may ultimately prove fruitful. Aminoguanidine displays greater specificity for iNOS than for cNOS (Misko *et al*., 1993) and it has been shown to cause partial reversal of vascular hyporeactivity (Joly *et al*., 1994; Wu *et al*., 1995), as well as improved survival rates in *in vivo* models (Wu *et al*., 1995). Several isothioureas, a class of potent NOS inhibitors, display some selectivity of action also (Southan *et al*., 1995) and also improve survival in animal models of septic shock (Szabo *et al*., 1994).

The design of drugs able to scavenge and remove pathophysiological quantities of NO, rather than prevent its synthesis, represents an alternative strategy for treating NO-mediated disease. We have explored the potential of a range of ruthenium compounds with this in mind and we report here on the pharmacological properties of two, chemically-related, polyaminocarboxylate-ruthenium(III) complexes: potassium chloro[hydrogen(ethylenedinitrilo)tetraacetato]ruthenate (or JM1226) and aqua[hydrogen(ethylenedinitrilo)tetraacetato] ruthenium (or JM6245).

A preliminary account of some aspects of this work was presented at the Fourth International Meeting on the Biology of Nitric Oxide (Fricker *et al*., 1996).

Methods

Binding of NO by aqueous solutions of JM1226

A known volume of authentic NO gas (3 cm^3; 1.3×10^{-4} moles) was introduced into the headspace above a stirred aqueous solution of JM1226 (25 cm^3; 1×10^{-4} moles) in a closed apparatus under an argon atmosphere at 22–24°C. NO absorption was measured using a manometer. After complete absorption of NO (about 20 min) the reaction mixture was freeze-dried and the product examined by infrared (IR) spectroscopy using a Perkin Elmer 1720X FT-IR spectrometer. In other experiments, equimolar amounts (9×10^{-5} M) of JM1226 and the NO donor SNAP were dissolved in water and allowed to react for 24 h. The resulting compound was freeze-dried and its IR spectrum recorded later.

Cell cultures

Cells were obtained from the European Collection of Animal Cell Cultures (ECACC, CAMR, Porton Down, Wiltshire, U.K.). Culture media were purchased from Imperial Laboratories (Andover, Hampshire, U.K.). The RAW 264 murine macrophage cell line (Raschke *et al*., 1978) was maintained in Eagle's minimal essential medium supplemented with 10% foetal calf serum, 1% non-essential amino acids, 100 iu ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin. P815 murine mastocytoma cells were grown in RPMI 1640 medium, supplemented with 10% foetal calf serum, 1 mM sodium pyruvate, 100 iu ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin. Cells were incubated at 37°C in an atmosphere of 5% CO_2/95% air.

Nitric oxide production by activated RAW 264 macrophages

RAW 264 cells were cultured on 24-well plates (2×10^6 cell/well) in 2 ml Eagle's minimal essential medium (but without phenol red). Cells were activated by the addition of 10 $\mu g\ ml^{-1}$ bacterial lipopolysaccharide (*E coli*, serotype 0111:B4; Sigma Ltd, Dorset, U.K.) and 100 IU ml^{-1} mouse recombinant IFNγ (Sigma Ltd). NO production was estimated 18 h later by assaying the medium for accumulated NO_2^-, the oxidation product of NO in an aqueous medium (Butler *et al*., 1995), using the Griess test (Green *et al*., 1982). Culture medium (1 ml) was added to Griess reagant (1 ml 1% sulphanilamide in 5% phosphoric acid plus 1 ml 0.1% naphthylethylenediamine dihydrochloride) and absorbance measured at 540 nm. NO_2^- accumulation was measured under the following conditions: (1) non-activated RAW 264 cells, (2) LPS/IFNγ-activated cells, (3) activated cells treated with JM1226 or JM6245 (100 μM), and (4) activated cells treated with L-NMMA (250 μM). Control experiments showed that neither JM1226 nor JM6245 was cytotoxic at the concentrations used in this study, as judged by the MTT assay (below). Cell viability was confirmed at the end of each experiment by adding 100 $\mu l\ ml^{-1}$ of 0.1% Neutral Red to the culture medium (2 h at 37°C) and examining the cells microscopically (Borenfreund & Puerner, 1985). Cell viability was not adversely affected by treatment with either JM1226, JM6245 or L-NMMA. Typically, the proportion of positive (red stained) cells exceeded 95%.

Macrophage tumour cell killing

The effect of JM1226 and JM6245 (100 μM) on tumour cell killing by macrophages was studied by co-culturing adherent LPS/IFNγ-activated RAW 264 cells (2×10^5 cells/well) with non-adherent P815 murine mastocytoma cells (5×10^4 cells/well) on 96-well plates. The final volume of culture medium was 200 μl. After 18 h, aliquots (125 μl) containing P815 cells in suspension were removed, incubated for 4 h with 75 μl 3-(4,5 dimethylthiazol-2-yl)-2,5 tetrazolium bromide (MTT) dissolved in Dulbecco's phosphate buffered saline (2 mg ml^{-1}, pH 7.4) and centrifuged at 1000 r.p.m. for 10 min. The insoluble blue formazan was dissolved in dimethylsulphoxide and the absorbance of the resulting solution measured at 540 nm (Mosmann, 1983). Results were compared with co-cultures of P815 cells with unstimulated RAW 264 cells which served as controls. NO_2^- was measured in the remaining supernatant using the Griess test. Results obtained with Ru compounds were compared with those obtained with the NOS inhibitor, L-NMMA (250 μM).

Isolated rat tail artery preparation

Vasodilator responses of the isolated rat tail artery preparation (Flitney *et al*., 1992) to SNAP were recorded in the presence and absence of JM1226 and JM6245. Male Wistar rats (330–420 g; $n = 11$) were killed by cervical dislocation. Segments of artery (10–12 mm) were dissected free, cannulated with a Portex cannula and perfused (2 ml min^{-1}) with pre-warmed Krebs solution (composition in mM: NaCl, 118; KCl, 4.7; $NaHCO_3$, 25; NaH_2PO_4, 1.15; $CaCl_2$, 2.5; $MgCl_2$, 1.1; glucose, 5.6; gassed with 95%O_2/5% CO_2 to maintain pH at 7.4; 32°C). Vessels were pre-contracted with phenylephrine (PE; 2–7 μM) to generate stable, active pressures (85–115 mmHg). Perfusion pressure was monitored using a differential transducer (Sensym SCX15ANC; Farnell Electronics, Leeds) and the output fed to a MacLab 4e A/D converter and stored for later analysis. Control microinjections (10 μl) of SNAP (10^{-7}–10^{-2} M)

were made into the perfusate immediately upstream of the vessel (delay time = about 6 s), producing transient (< 5 min) vasodilator responses (Askew *et al*., 1995). SNAP injections were repeated after adding JM1226 or JM6245 (100 μM) to the perfusate, and then again 1 h after washout. Results of experiments with JM1226 and JM6245 were compared with those obtained by adding 5 μM oxyhaemoglobin (Hb) to the perfusate.

Effects of JM1226 on haemodynamics in a rat model of endotoxic shock

Male Wistar rats (250–340 g) were given a single injection of bacterial lipolysaccharide (4 mg kg^{-1}; i.p., *E.coli* serotype 055:B5; Sigma Ltd). The time course of the development of endotoxaemia was monitored as follows:

First, by measuring blood pressure before and after LPS treatment in conscious rats, using a tail cuff apparatus (Model 179 Blood Pressure Analyzer, IITC Life Science, CA., U.S.A.). Recording of systolic (SAP), mean (MAP) and diastolic (DAP) arterial pressures were made at the following times relative to the time (t = 0 h) at which animals were injected with LPS; t = 0, 20, 24, 29 and 32 h.

Second, by monitoring the accumulation of NO_2^- and NO_3^- levels (= $NO_\times$) in the plasma at various times (t = 4, 6, 12, 18, 24, 48 and 72 h) after LPS injection. Animals were sacrificed by cervical dislocation and exsanguinated. The main oxidation product of NO in blood is NO_3^- (Wennmalm *et al*., 1992) and this was enzymically reduced to NO_2^- before performing the Griess test. Whole blood (5–8 ml) was centrifuged (15 000 r.p.m., 10–15 min) and 2 ml aliquots of plasma freeze-dried overnight. Samples containing high $NO_\times$s (t = 18, 24 and 48 h) were re-constituted in 8 ml de-ionized water (4 × dilution) and those with low $NO_\times$s (from control animals and LPS-treated animals at t = 4, 6, 12 and 72 h) in 0.5 ml de-ionized water (4 × concentrated). Nitrate reductase from *Aspergillus spp* (Boehringer Mannheim, Lewes, U.K.) was used to convert NO_3^- to NO_2^-. Two sets of five sample tubes were used, each containing reconstituted plasma (127 μl), to which the following were added (final concentrations): FAD (48.2 μM), NADPH (482 μM), phosphate buffer (pH 7.4; 40 mM), nitrate reductase (70 mU) and, in tubes 1–5, respectively, the following quantities of Analar grade authentic NO_3^- (set 1) or NO_2^- (set 2) as 'internal' standards: 45, 22.5, 15.75, 11.25 and 2.25 nmoles. Tubes were incubated for 2 h at 37°C before performing the Griess test. Linear plots of absorbance against added NO_2^- were obtained, with negative intercepts on the abscissa, representing apparent $NO_\times$ in the plasma sample. A correction was made to allow for the efficiency of enzymatic reduction of NO_3^- to NO_2^- (generally > 95% conversion).

Third, the responsiveness of isolated tail arteries to bolus (10 μl) injections of PE (10^{-5}–10^{-2} M) was monitored at various times after injecting animals with LPS (t = 6, 12, 18, 24 and 72 h). PE injections into perfused vessels (not pre-contracted) produced transient vasoconstrictor responses. Peak pressures were measured and used to construct log dose–response curves for arteries perfused with (1) Krebs' solution only; and (2) Krebs' solution containing JM1226 or JM6245.

Fourth, Western blots were made from extracts of lung, heart, liver and tail arteries taken from animals sacrificed at different times after LPS injection (t = 4, 6, 12, 24 and 72 h). Tissues were homogenized for 3–5 min at 24 000 r.p.m. (Ultraturrax T25, Janke & Kunkel, IKA Lab Tech.) in 5 volumes ice cold lysis buffer (composition: 0.05 M; Tris(hydroxymethyl)methylamine plus 0.001 M EDTA, containing: 0.001 M dithiothreitol; 0.001 M phenylmethylsulphonyl fluoride; 10 μg ml^{-1} trypsin inhibitor; 1 μg ml^{-1} antipain and 1 μg ml^{-1} pepstatin A). The resulting lysate was centrifuged

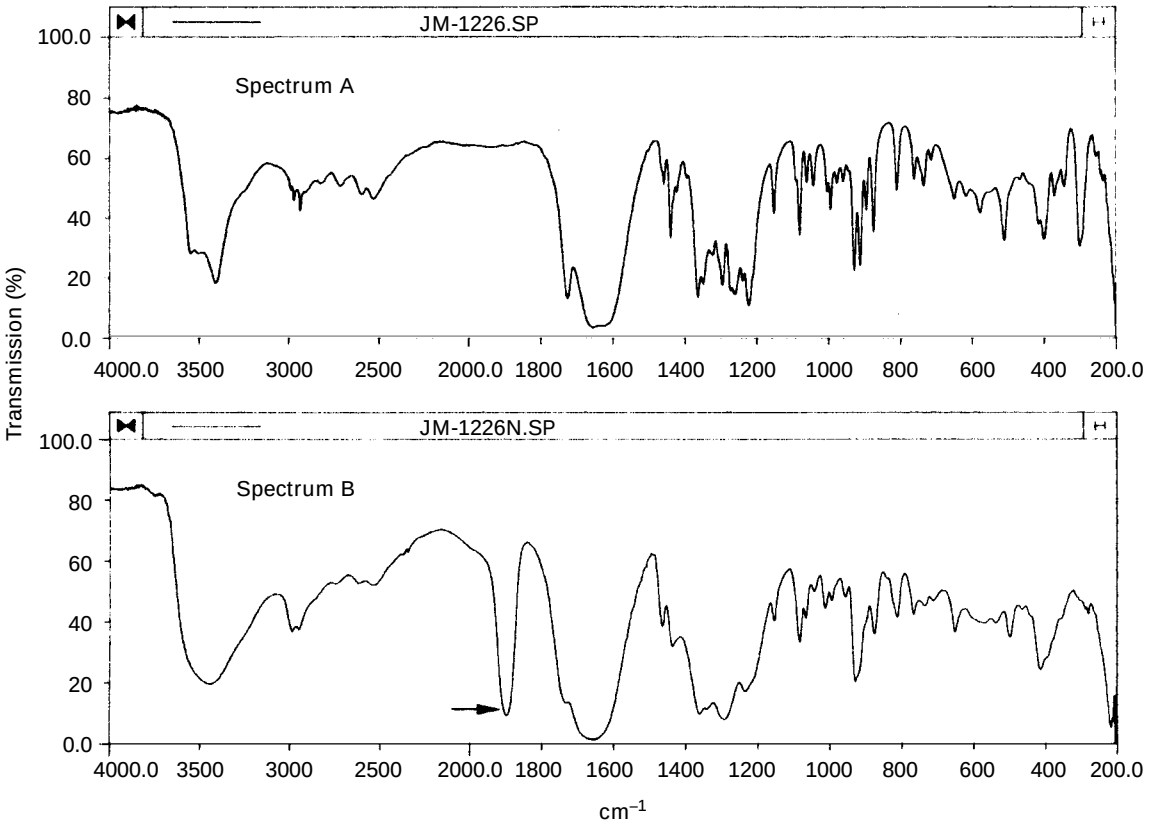

Figure 1 Infrared spectra of JM1226 (A) and JM1226 after reaction with NO (B). The product has a sharp absorbance peak at 1897 cm^{-1} (arrow), characteristic of a linear Ru(II)-NO adduct. Reaction of JM1226 with SNAP gave a product with a similar spectrum (data not shown).

(100 000 *g*, 1 h, 4°C) and the protein content of the supernatant estimated using the Bradford assay. Samples containing 2 mg protein. ml^{-1} (lung, liver and heart) or 1 mg protein ml^{-1} (tail arteries) were mixed with 0.5 × volume of Laemmli buffer (3 × strength) and β-mercaptoethanol added to a final concentration (v/v) of 15%. Samples were boiled for 5–10 min and subjected to SDS-polyacrylamide gel electrophoresis (15 μl per well; 7.5% SDS). Proteins were transferred onto a nitrocellulose membrane, probed overnight with a rabbit polyclonal antibody (1 : 1000 dilution) raised against a synthetic murine macrophage iNOS polypeptide (Hamid *et al*., 1993), and afterwards treated with goat anti-rabbit IgG-horseradish peroxidase secondary antibody (Scottish Antibody Production Unit, Lanarkshire, Scotland; 1 : 3000 dilution). Secondary antibody was detected using ECL Western blotting detection reagants and Hyperfilm ECL (Amersham International, Bucks, England). Boehringer Combithek molecular weight markers (about 26–170 K) were used to estimate apparent molecular weights of iNOS positive bands.

Statistical analyses

All quantities are expressed as mean values ± s.e.mean. Two way ANOVA was used to compare log dose–response curves. Blood pressures, NO_x measurements and tumour cell viability were analysed using the unpaired, two-tailed Student's *t* test. Results were considered statistically significant when $P<0.05$.

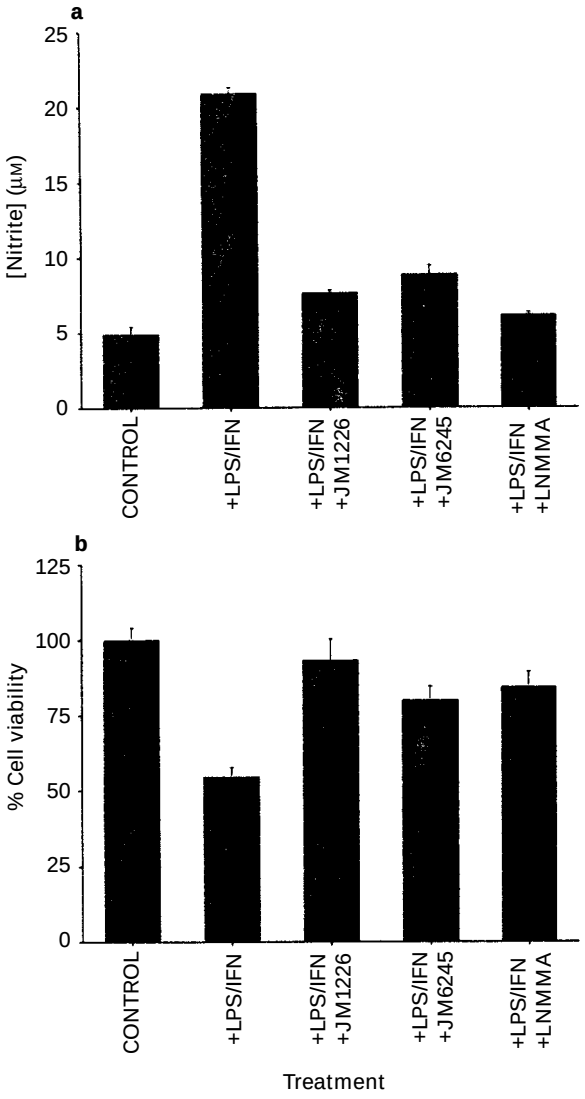

Figure 2 (a) Effect of JM1226 and JM6245 on nitrite production by activated RAW 264 macrophages in culture. Column (left to right) 1: unactivated RAW 264 cells; 2: RAW 264 cells after activation with LPS + IFNγ; 3: activated RAW 264 cells cultured for 18 h in presence of JM1226 (100 μM); 4: activated RAW 264 cells cultured for 18 h in presence of JM6245 (100 μM); 5: activated RAW 264 cells cultured for 18 h in presence of L-NMMA (250 μM). JM1226, JM6245 and L-NMMA all significantly reduced nitrite accumulation to near control levels. (b) Effect of JM1226, JM6245 (100 μM) and L-NMMA (250 μM) on the viability of P815 mastocytoma cells co-cultured with LPS + IFNγ-activated RAW 264 macrophages. Tumour cell viability was reduced following activation of RAW 264 cells (columns 1 and 2). Significant protection was afforded by JM1226 (column 3) and JM6245 (column 4) and also by blocking NO synthesis with L-NMMA (column 5).

Results

JM1226 binds NO to form a ruthenium (II) mononitrosyl

Figure 1 shows FT–IR spectra for JM1226 alone (A) and for JM1226 after reacting it with authentic NO in aqueous solution (B). The sharp absorbance peak at 1897 cm^{-1} in spectrum B (arrow) identifies a linear Ru(II)-NO bond in the resulting compound (Bottomley, 1978) and provides confirmation of the formation of a ruthenium(II) mononitrosyl. A similar result (spectrum not shown) was obtained after reacting JM1226 with the NO donor SNAP.

JM1226 and JM6245 reduce NO_2^- accumulation in the culture medium of activated RAW 264 macrophages

Figure 2a compares NO_2^- levels in media from cultures of non-activated RAW 264 cells (control levels) and LPS + IFNγ-treated RAW 264 cells, with media from cultures of activated RAW 264 cells treated with either JM1226 or JM6245 (both 100 μM) or L-NMMA (250 μM). Combined LPS + IFNγ treatment produced a four fold increase in NO_2^- accumulation compared with untreated cultures. Control ex-

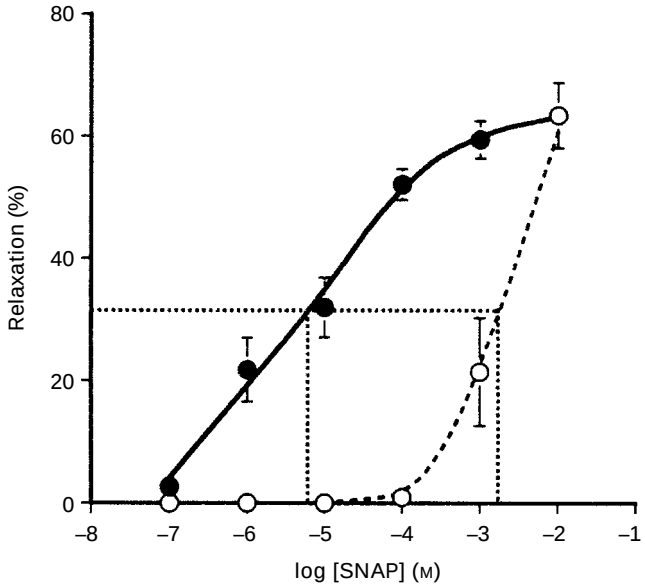

Figure 3 Vasodilator responses of PE-precontracted, perfused tail arteries elicited by bolus injections (10 μl) of SNAP. Vessels were perfused with Krebs' solution only (●) or with Krebs' solution containing 100 μM JM6245 (○). JM6245 strongly attenuated responses to SNAP, producing a rightward shift of the log dose–response curve ($P<0.001$).

periments showed that this was prevented by co-administration of actinomycin D (10 μM) or cycloheximide (5 μM), although neither drug was effective when added 6 h after LPS + IFN-γtreatment.

JM1226 and JM6245 significantly reduced NO_2^- levels in growth media of activated cells. A comparable reduction was observed following treatment with L-NMMA (250 μM). In the case of all three compounds, NO_2^- levels were similar to those obtained in media from unactivated macrophages.

JM1226 and JM6245 protect P815 tumour cells against activated RAW 264 cells

Figure 2b shows that P815 tumour cell viability decreased following activation of RAW 264 macrophages ($P < 0.001$). The addition of 100 μM JM1226 or JM6245 to the culture medium of stimulated cells had a protective effect and raised tumour cell viability ($P<0.05$). The degree of protection afforded was the same as that seen after blockade of NO production by L-NMMA (250 μM: $P>0.05$ for JM1226 vs L-NMMA and also for JM6245 vs L-NMMA).

JM1226 and JM6245 attenuate vasodilator responses of isolated rat tail arteries to SNAP

Figure 3 shows log dose–response curves to successive injections of increasing concentrations of SNAP delivered into (a) arteries perfused with Krebs' solution only (●) and (b) arteries perfused with Krebs' solution containing 100 μM JM6245 ($n=6$). JM6245 markedly attenuated SNAP-induced vasodilator responses, producing a large rightward shift of the log dose–response curve: the ED_{50} value increased 300-fold, from 6 μM to 1.8 mM. A qualitatively similar result was obtained in a seperate series of experiments using 100 μM JM1226: in this case, the ED_{50} increased approximately 20 fold, from 7 μM in Krebs' solution to 132 μM in Krebs' solution + JM1226. For comparison,

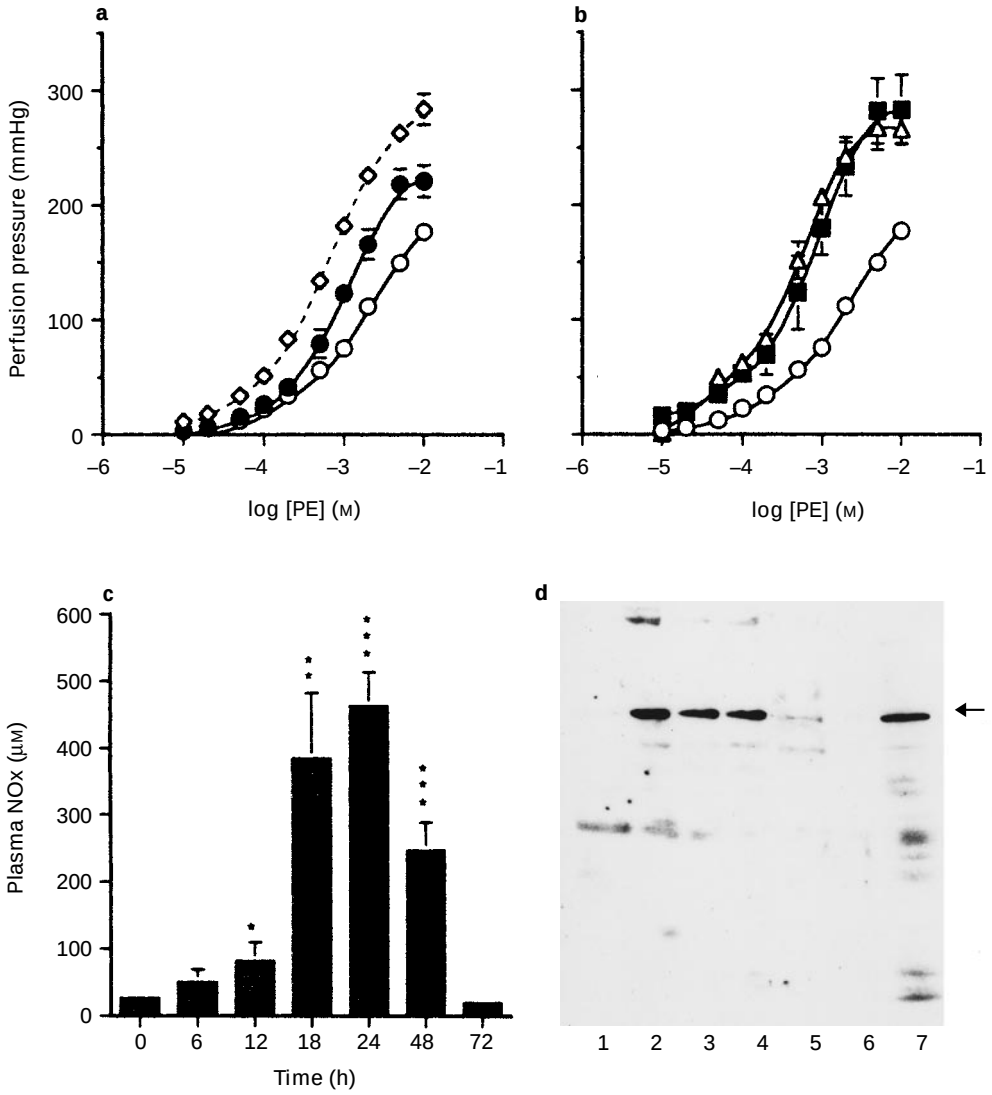

Figure 4 (a) Log dose–response curves to PE showing vasoconstrictor responses of isolated, perfused arteries from LPS treated rats (●, t = 18 h; ○, t = 24 h) compared to vessels from control animals which received an injection of sterile saline only (- - -; ◇). Vessels from both groups of LPS-treated animals were significantly less responsive to PE ($P<0.001$). (b) Log dose–response curves to PE of vessels taken from LPS-treated animals at t = 48 h (△) and t = 72 h (■) compared to arteries from LPS-treated rats sacrificed at t = 24 h (○). Data for control (Figure 4a, - - -), t = 48 and t = 72 h vessels are not significantly different ($P>0.05$). (c) Changes in plasma NO_x levels measured at different times after a single injection of LPS. NO_x was maximal after 18–24 h and returned to control levels over the next 48–54 h. (d) Western blot of tail artery lysates (lanes 1–6) and of a rat lung lysate (lane 7) probed with a rabbit polyclonal antibody to macrophage iNOS. Lanes 2–6 tail artery lysates from LPS-treated rats (t = 4, 6, 12, 24 and 72 h, respectively), compared to tail arteries from control (i.e. saline injected) animals (lane 1) and with lung (lane 7) from an LPS-injected animal (t = 24 h). The iNOS signal (arrow, apparent M_r, 133 kDa) was strong at t = 4–12 h, less so at t = 24 h and had disappeared by t = 72 h.

oxyhaemoglobin (5 μM) increased the ED_{50} for SNAP from 8 μM to 200 μM. The attenuating effect of both Ru compounds was long-lasting: a 1 h washout period in Krebs' solution alone failed to fully reverse the action of either compound.

LPS-induced endotoxaemia in an experimental rat model

Table 1 shows that a single i.p. injection of LPS (4 mg kg^{-1}) induced a marked hypotensive response in experimental rats ($n=13$): at t = 20 h, SAPs, MAPs and DAPs had fallen by 32%, 28.5% and 25.1%, respectively. Systemic pressures spontaneously returned to preinjection values over the ensuing 24–28 h (t = 44–48 h). LPS also induced a modest (about 10%) but statistically significant tachycardia.

Perfused tail arteries ($n=13-16$ vessels) taken from animals sacrificed at different times after administering LPS exhibited a progressive decline in sensitivity to PE compared with those from control animals which received an injection of sterile saline instead (Figure 4a). The hyporesponsiveness to PE was statistically significant ($P<0.001$) by t = 18 h and maximal at t = 24 h. Vessels from animals injected with LPS and left for >24 h before sacrifice were less hyporeactive (Figure 4b) and by t = 48 and 72 h their sensitivity to PE was not significantly different from that of arteries taken from control animals.

Figure 4c shows that the diminished sensitivity to PE (Figure 4a) and the associated hypotension (Table 1) were accompanied by an 18-fold increase in plasma NO_2^- and NO_3^- (NO_x). NO_x levels were maximal at t = 24 h, then returned to preinjection levels over the ensuing 48 h (t = 72 h).

Western immunoblots of tail artery homogenates from LPS-treated and control animals revealed time-dependent changes in iNOS expression. The signal was strong at t = 4, 6 and 12 h, then declined until it was barely detectable by t = 24 h and was absent by t = 72 h (Figure 4d).

JM1226 and JM6245 reverse the hyporeactivity to PE of tail arteries from LPS-treated rats

Figure 5a compares vasoconstrictor responses to PE of isolated, perfused tail arteries from LPS-treated animals ($t=24$ h) with vessels from control animals ($n=9$). Vessels from LPS-treated animals were significantly less responsive to PE ($P<0.001$). The addition of JM1226 (100 μM) to the internal perfusate increased the sensitivity of LPS-treated arteries (Figure 5b; $P<0.001$). The effect of JM1226 (and of JM6245; data not shown) on vessels from LPS-treated rats was comparable to that seen with L-NMMA (100 μM, Figure 6b). Statistically, there was no significant difference between JM1226-, JM6245- and L-NMMA-treated arteries from LPS-treated rats ($P>0.05$).

JM1226 accelerates recovery of arterial blood pressures in LPS-treated (hypotensive) rats

Two types of experiment were made in which blood pressures were measured in conscious rats at various times after administering a single i.p. injection of LPS (4 mg kg^{-1}), followed later (at t = 20 h) by a second injection of either (1) sterile saline (group 1) or (2) a sterile solution (100 mg kg^{-1}) of JM1226 (group 2). In the first experiment, blood pressures were measured at t = 0, 20 and 24 h. In the second experiment, recordings were made at t = 0, 20, 24 h and also at t = 29 and 32 h. The results are shown in Figure 6. The data from experiments 1 and 2 are combined for time points t = 0, 20 and 24 h, but for time points t = 29 and 32 h the data is from experiment 2 only.

Both groups of LPS-treated animals were markedly hypotensive by t = 20 h (see Table 1 which shows combined data, i.e. both groups, two experiments: $n=13$ animals) and showed some recovery thereafter. However, the recovery of saline-injected animals was incomplete even after 12 h (t = 32 h), whereas the rate of recovery of animals that received JM1226 was enhanced and essentially complete within about 9 h (t = 29 h).

Table 1 Haemodynamic parameters of endotoxin-treated rats

	Time after injection	
	t = 0 h	*t = 20 h*
SAP	166.4 ± 8.8	113.2 ± 5.2
MAP	110.6 ± 4.9	79.1 ± 6.4
DAP	82.8 ± 4.9	62.0 ± 7.8
HR	376.6 ± 9.4	411.1 ± 11.7

Systemic blood pressures (mmHg) and heart rates (HR; beats min^{-1}) recorded with the tail cuff apparatus immediately before and 20 h after injecting rats ($n=13$) with bacterial LPS (4 mg kg^{-1}). Student's t test showed that all haemodynamic parameters for LPS-treated animals were significantly different from untreated animals ($P<0.02$).

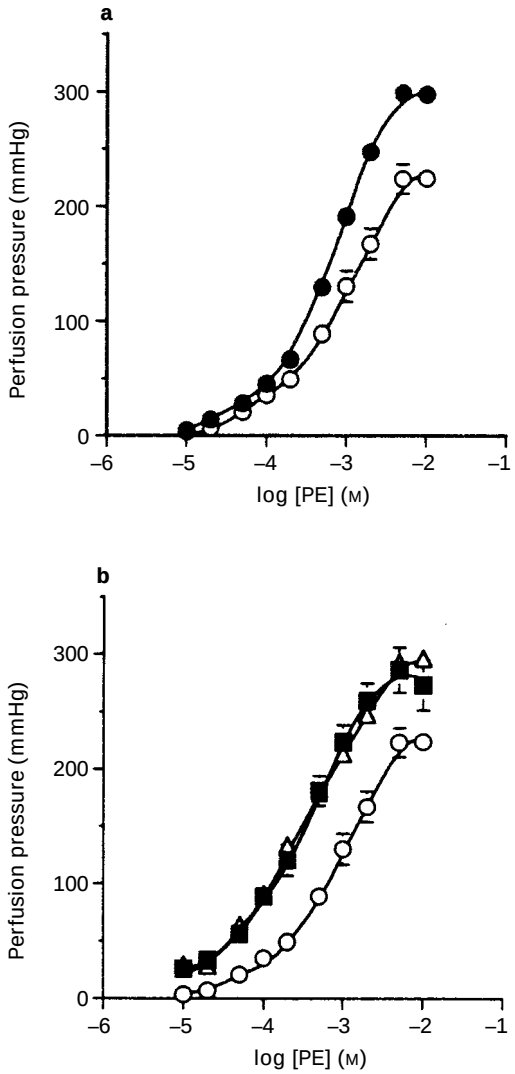

Figure 5 (a) Log dose–response curves showing the vasoconstrictor effect of bolus injections of PE on arteries isolated from animals previously injected with LPS 24 h prior to sacrifice (○) compared to vessels from animals injected with sterile saline only (controls; ●). (b) Log dose–response curves to PE of vessels from LPS-treated rats (t = 24 h) before (○; same data as in Figure 6a) and after the addition of either JM1226 (■) or L-NMMA (△) to the internal perfusate. The sensitivities to PE of L-NMMA and JM1226-treated vessels were identical and both significantly greater than that for LPS-treated arteries.

Discussion

Ruthenium forms more nitrosyl complexes than any other metal (Bottomley, 1978). Ru(III) reacts rapidly with NO to form six coordinate Ru(II) mononitrosyls containing a linear Ru-NO bond. The latter is very stable (Davies *et al*., 1997), able to resist a variety of redox and substitution reactions and consequently the nitrosyl moiety is not easily displaced. Chelation of the metal with a suitable ligand can be used to confer water solubility, facilitating rapid *in vivo* clearance and low toxicity, while providing an available binding site for NO. The polyaminocarboxylates, such as ethylenediaminetetraacetic acid (EDTA), satisfy these requirements as ligands. In both complexes studied here the EDTA is pentadendate with one coordination position available for NO binding.

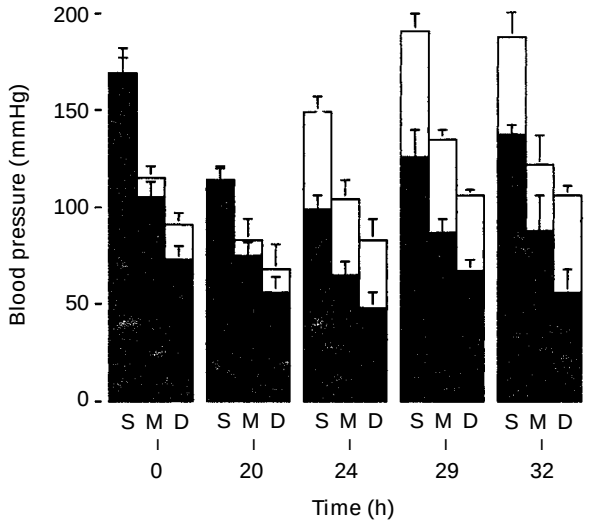

Figure 6 Haemodynamic responses of rats to LPS and LPS + JM 1226. Each of the five grouped columns in the histogram represents (left to right) SAPs (S) MAPs (M) and DAPs (D). Blood pressure measurements are shown for rats immediately before (t = 0 h; first block) and 20 h after (second block) a single i.p. injection (4 mg kg^{-1}) of bacterial LPS (data also shown in Table 1). Animals in group 1 (■) received an injection of saline only and those in group 2 (□) received JM1226 (i.p.; 100 mg kg^{-1}) at t = 20 h. Blood pressures recovered more rapidly in animals treated with JM1226 and was essentially complete after approximately 9 h.

The reaction of JM1226 with NO in aqueous solution involves formation of the aqua derivative, JM6245, followed by rapid substitution of H_2O for NO (Figure 7). The substitution proceeds by associative ligand attack (Bajaj & van Eldik, 1990) via a seven coordinate intermediate. The binding of NO by JM1226/6245 is extremely rapid ($k = 2 \times 10^7$ M^{-1} s^{-1} at pH 7.4 and 7.3°C; Davies *et al*., 1997). The EDTA ligand around ruthenium(III) is kinetically inert and formation of the Ru-NO bond further stabilises the *trans* position in the resulting ruthenium(II) adduct.

This study suggests that ruthenium-based NO scavengers offer an alternative therapeutic strategy to the use of NOS inhibitors for alleviating some of the symptoms of septic shock. Both JM1226 and JM6245 reacted with authentic NO and also with an NO donor (SNAP) in aqueous solution to yield the corresponding Ru(II) mononitrosyl adduct. Their ability to scavenge NO in several biological systems of increasing complexity (from cultured cells to whole animals) was therefore explored, following activation of the iNOS-driven, 'high-output' pathway for NO synthesis, using either LPS alone (whole animals) or combined LPS + IFNγ treatment (cell cultures).

Both ruthenium complexes reduced NO_2^- accumulation by activated macrophages in culture. JM1226 and JM6245 were also able to afford a significant degree of protection against the NO-mediated tumouricidal action of activated macrophages when tested with the non-adherent P815 mastocytoma line as the 'target' cell. Moreover, they were shown to markedly attenuate the vasodilator action of SNAP in experiments using the isolated, pre-contracted rat tail artery preparation. All of these effects are consistent with a 'scavenging' role for JM1226 and JM6245.

The results of *in vivo* experiments using the rat model of endotoxic shock are of more relevance when evaluating the

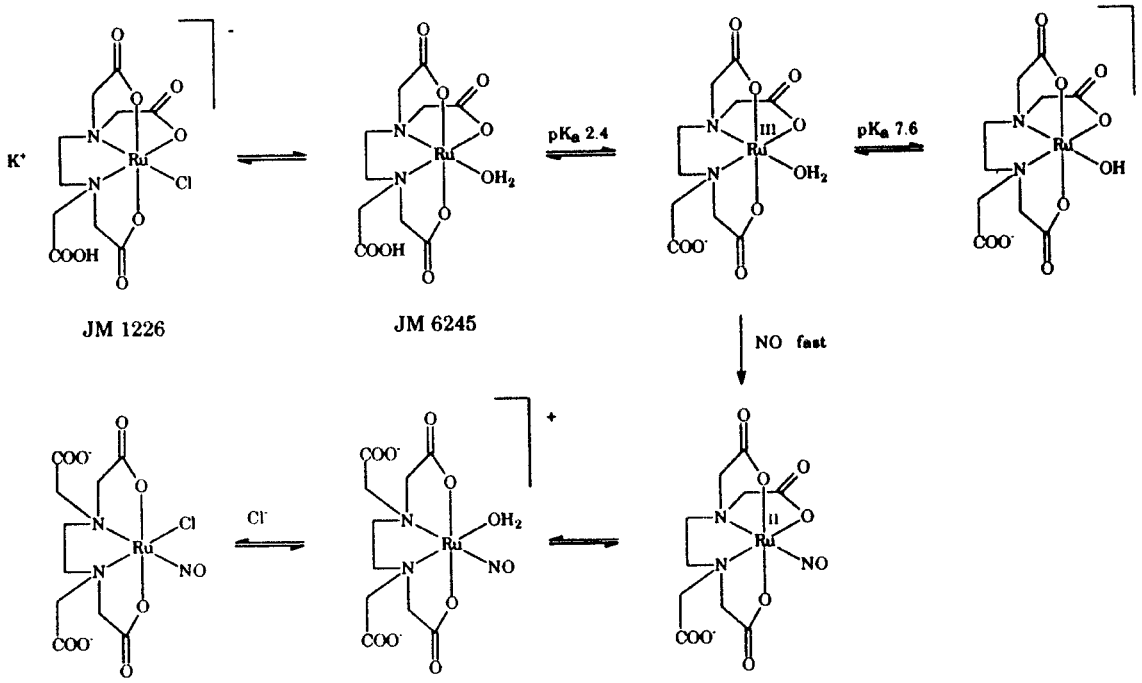

Figure 7 Reaction between JM1226 and NO in aqueous solution. Reaction between the aqua derivative, JM6245, and NO is rapid and proceeds via a seven coordinate intermediate. The resulting adduct contains a stable, linear Ru(II)-NO bond (See Figure 1).

therapeutic potential of these compounds. We selected a moderate dose of LPS (4 mg kg^{-1}) to induce shock, with the aim of minimising mortality rates. The severe hypotension resulting from LPS treatment, accompanied by a marked vascular hyporesponsiveness to PE, expression of iNOS protein and elevated NO production, as judged by the accumulation of its stable oxidation end-products in plasma (Tracey *et al*., 1995), are all consistent with the principal physiological manifestations of septic shock (Glauser *et al*., 1991) and they establish our LPS-treated rats as a valid paradigm for testing the ability of JM compounds to scavenge pathophysiological quantities of NO under both *ex vivo* (isolated arteries) and *in vivo* conditions.

The hyporesponsiveness to PE of perfused rat tail arteries isolated from LPS-treated animals was maximal 24 h after injection of LPS. The addition of JM1226 to the perfusate of such vessels greatly enhanced their sensitivity to PE: indeed, the effect was identical to that seen after blockade of NO synthesis at source, using the NOS inhibitor L-NMMA.

Treatment with LPS produced a profound hypotensive response in laboratory rats. SAPs, MAPs and DAPs fell to a minimum at around t = 20 h, then gradually recovered over the ensuing 24–28 h (t = 44–48 h). The time course of the hypotensive effect was comparable with that observed for the development of vascular hyporeactivity and for increased plasma NO_x levels. iNOS expression, as judged by Western immunoblots of tail artery extracts, preceded all three: immunoreactivity was strongest 4–6 h after LPS injection, substantially reduced by t = 24 h and below the limit of detection by t = 72 h). Some spontaneous recovery of blood pressures was seen in the saline-injected (control) group of LPS-treated rats but this was clearly incomplete after 12 h (t = 32 h). In contrast, the recovery of the LPS-treated group that received a single i.p. injection of JM1226 given at t = 20 h was accelerated and complete after a further approximately 9 h (t = 29 h).

These results show that JM1226 and JM6245 are effective scavengers of NO in a variety of biological test systems and they establish these complexes as potentially useful alternatives to NOS inhibitors for alleviating some of the symptoms of endotoxic shock. Other, equally important pathophysiological conditions exist where the potential benefits of such compounds might be usefully explored. For example, tumour supply vessels are known to be hyporesponsive to vasoactive agents (Peterson, 1991; Hirst & Tozer, 1992) and in this respect they strongly resemble tail arteries from LPS-treated rats. We have shown that the sensitivity to PE of isolated, perfused epigastric arteries which previously supplied an inguinal tumour implant can be fully restored using the non-selective NOS inhibitor, L-NAME (Kennovin *et al*., 1994). Moreover, in the same study we demonstrated that chronic, oral administration of L-NAME via the drinking water significantly retarded solid tumour growth. Based on the data presented here we anticipate that JM1226 and JM6245 might have similar growth-retarding effects as L-NAME.

The possibility that some of the undesirable effects of therapeutic interventions which employ non-selective NOS inhibitors, referred to earlier (Introduction), might be avoided by using NO scavengers should be explored in future studies. Detailed toxicological and pharmacokinetic studies of JM1226 and JM6245 have yet to be made. It is worth re-emphasising here that neither compound was found to be cytotoxic in our *in vitro* experiments with cultured macrophages and P815 mastocytoma cells (12.5–200 μM range, for 18 h) nor did we observe any obvious deleterious side-effects when JM1226 was used *in vivo*, over and above those attributable to LPS treatment alone. Preliminary toxicity tests *in vivo* have shown that Wistar rats can tolerate maximum doses of JM1226 some two to four times greater than those used in the present study and pilot pharmacokinetic experiments indicate rapid plasma clearance times.

In conclusion, the results of this study demonstrate that ruthenium(III) polyaminocarboxylate complexes are able to scavenge NO in several models of differing pathophysiological complexity and we suggest that these compounds might be usefully exploited in designing novel therapeutic strategies aimed at combatting NO-mediated disease.

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