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Synthesis, pharmacological activity and nitric oxide generation by nitrate derivatives of theophylline

Lucyna Korzycka and Dorota Górska

Abstract

Nitrates of theophylline derivatives – potential nitric oxide (NO) donors – were synthesized by esterification of 7-hydroxyalkyl theophylline derivatives with fuming nitric acid. The nitrates obtained were tested in-vitro in reactions with sulfydryl compounds at appropriately adjusted pH and temperature. Under the applied conditions, the synthesized compounds underwent decomposition to release NO, quantified using a polarographic method using a selective isolated (ISO-NO) sensor. The effects of dyphylline and proxyphylline and their new synthesized nitrates on arterial blood pressure (BP) were measured in spontaneously hypertensive (SH) rats. BP was measured in conscious SH rats using the tail-cuff method. Both short- and long-term administration of the xanthines tested significantly decreased systolic, diastolic and mean BP. The hypotensive effect of a single dose of nitrate dyphylline on mean BP was greater than that of the parent compound ($P = 0.000012$; $P = 0.000472$ at 30 and 60 min post-dose, respectively), whereas proxyphylline and its nitrate derivative had similar activity. In rats treated with the tested compounds for 9 days twice daily, the decrease in BP persisted for at least 16 h after the last dose. Proxyphylline produced the most marked decrease in diastolic and mean BP. Among the xanthines examined, proxyphylline nitrate had the strongest hypotensive effect when administered in a single dose to animals pretreated with the same compound for 9 days. These results indicate that insertion of a nitrate group weakly modifies the hypotensive action of the studied xanthines in SH rats.

Introduction

Theophylline, a methylxanthine derivative, is a weak and non-selective inhibitor of phosphodiesterases (PDEs) of cyclic nucleotides, leading to an increase in intracellular concentrations of cyclic AMP and cyclic GMP (Barnes 2003). The other well-described pharmacological action of the drug is its potent antagonistic activity at adenosine receptors. Theohylline has also been shown to increase release of interleukin-10 (presumably by a mechanism involving inhibition of PDE), antagonizes tumour necrosis factor alpha, inhibits the pro-inflamatory effects of prostaglandins, decreases release of intracellular calcium ions, prevents the translocation of nuclear factor-κB into the nucleus (and thus potentially reduces the expression of inflammatory genes) and increases apoptosis and histone deacetylase activity (Barnes 2003). The multiple mechanism of action of theophylline is one of the major factors responsible for the various actions that the drug exerts on the circulatory system. Dose-dependent tachycardia and an increase in systolic blood pressure (BP) were observed in healthy human subjects after intravenous injection of theophylline. Theophylline has also been found to increase plasma adrenaline and noradrenaline levels (Vestal et al 1983). On the other hand, oral theophylline exerted a small effect on plasma catecholamines (Higbee et al 1982). Increase of mean arterial BP and heart rate after administration of methylxanthines have been related to enhanced release of catecholamines and blockade of adenosine receptors. Edlund et al (1995) suggested that the theophylline-induced increase in coronary vascular resistance in man could also result from the ability of the drug to block adenosine receptors. Theophylline attenuated the increase in heart rate and systolic BP after intravenous administration of adenosine (Biaggioni et al 1991). Crea et al (1989) showed beneficial effects of a single oral dose of theophylline on coronary vessels in a double-blind placebo-controlled trial. The drug was able to redistribute coronary blood flow from non-ischaemic to ischaemic myocardium. In studies of interactions between inhaled fenoterol and oral theophylline, the haemodynamic effects of

Department of Pharmaceutical Chemistry and Drug Analysis, Faculty of Pharmacy, Medical University of Lodz, Muszynskiego 1, PL 90-151 Lodz, Poland

Lucyna Korzycka

Department of Pharmacodynamics, Faculty of Pharmacy, Medical University of Lodz, Muszynskiego 1, PL 90-151 Lodz, Poland

Dorota Górska

Correspondence: L. Korzycka, Department of Pharmaceutical Chemistry and Drug Analysis, Faculty of Pharmacy, Medical University of Lodz, Muszynskiego 1, PL 90-151 Lodz, Poland. E-mail: korzycka@pharm.am.lodz.pl

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this methylxanthine were no different from those of placebo in healthy subjects (Flatt et al 1989). Theophylline infusion in patients after elective coronary artery bypass grafting did not affect cardiac arrhythmias, BP or heart rate (Krämer et al 2002).

Theophylline also induces diuresis and natriuresis as a consequence of adenosine A_1 receptor blockade. Methylxanthines increase excretion of renal water and sodium, mainly by affecting tubular reabsorption (Rieg et al 2005). This action may lead to a decrease in arterial BP.

All xanthine derivatives with 7-position substituents, such as dyphylline and proxyphylline, were found to be weaker antagonists of A_1 and A_2 receptors than xanthine (Schwabe et al 1985). Dyphylline decreased pulmonary vascular resistance in patients with chronic pulmonary disease by markedly reducing the mean pulmonary arterial pressure. However, it did not significantly change the cardiac output in stable patients (Kang 1992). Dyphylline did not significantly affect the heart rate in horses (Ayres 1985). Dyphylline, in contrast to theophylline and proxyphylline, had no positive inotropic effects on guinea-pig atria (Kukovetz et al 1983). Theophylline and proxyphylline have been shown to inhibit tracheal PDE activity with a similar potency, whereas dyphylline was about 5 times less effective (Kukovetz et al 1983). In isolated dog heart, proxyphylline increased coronary blood flow, heart rate and myocardial contractile force (Sakai et al 1978).

The available literature provide somewhat contradictory information about the effects of methylxanthines on the circulatory system. The aim of our study was to investigate the effects of dyphylline and proxyphylline and their newly synthesized nitrates on arterial BP in spontaneously hypertensive (SH) rats.

Materials and Methods

Chemical studies

Melting points were measured on a Boetius apparatus (VEB Wägetechnik Rapido, Dresden, Germany) and are given uncorrected. Infrared (IR) spectra were taken in KBr using a Mattson Infinity MI-60 Fourier transform-IR spectrophotometer (Madison, WI, USA). ^{1}H-NMR spectra were recorded on a Mercury-300 MHz (Varian, Inc., Palo Alto, CA, USA), using d_6-DMSO as the solvent and tetramethylsilane as the internal reference. Carbon, hydrogen and nitrogen elemental analyses were performed using a Perkin Elmer 2400 series II CHNS/O analyser (Norwalk, CT, USA) and agreed with the proposed structures within ±0.3% of theoretical values.

Synthesis of 7-(2-hydroxy-3-nitrooxypropyl) theophylline (1) and 7-(2-nitrooxypropyl) theophylline (2)

Dyphylline (7-(2,3-dihydroxypropyl)theophylline, **1a**) and proxyphylline (7-(2-hydroxypropyl)theophylline, **2a**) were purchased from Sigma-Aldrich (St Louis, MO, USA). Compound **1** (dyphylline nitrate, 7-(2-hydroxy-3-nitrooxypropyl) theophylline) was obtained from the reaction of compound **1a** with fuming nitric acid. Esterification was conducted at −5°C, with the reagents being stirred intensively and continuously: 0.025 mol fuming nitric acid was added slowly (so as not to allow the reaction temperature to rise above −5°C) to 0.02 mol compound **1a** dissolved in 10 mL ice-cold acetic acid. The reaction was continued at 0°C for 1 h. After that time, 30 mL chloroform was added to the reaction mixture, and its pH value was adjusted to 6.0 with aqueous sodium bicarbonate solution cooled to 0°C. The chloroform fraction was separated and dried with anhydrous magnesium sulfhate. After distillation of chloroform under reduced pressure, the product obtained was crystallized from ethyl acetate, and then from ethanol.

Yield: 24.9%; m.p. 146–148°C; ^{1}H-NMR (ppm): δ: 3.229 (s, 3H, CH_3); 3.431 (s, 3H, CH_3); 3.787–3.822 (m, 2H, CH_2); 4.033–4.107 (m, 1H, CH); 4.391–4.436 (d, 2H, CH_2); 5.006–5.025 (d, 1H, OH); 7.968 (s, 1H, =CH). IR (cm^{-1}): 3327 (ν OH); 1696, 1657 (ν C=O); 1636, 1281 (ν ONO_2). Analysis: calculated for $C_{10}H_{13}N_5O_6$ (299.24): C, 40.14%; H, 4.38%; N, 23.40%. Found: C, 40.26%; H, 4.39%; N, 23.46%.

Compound **2** (proxyphylline nitrate, 7-(2-nitrooxypropyl) theophylline) was obtained by esterification of compound **2a** with fuming nitric acid in a 1:3 molar ratio, at −10°C. The synthesis was as described above for compound **1**. Crude compound **2** was crystallized from ethyl acetate.

Yield: 65.3%; m.p. 132–134°C; ^{1}H-NMR (ppm): δ: 3.240 (s, 3H, CH_3); 3.446 (s, 3H, CH_3); 3.804–3.834 (d 3H CH_3); 4.062–4.112 (m, 1H, CH); 4.402–4.447 (d, 2H, CH_2); 8.042 (s, 1H, =CH). IR (cm^{-1}): 1690, 1651 (νC=O); 1628, 1271 (νONO_2). Analysis: calculated for $C_{10}H_{13}N_5O_5$ (283.24): C, 42.41%; H, 4.63%; N, 24.72%. Found: C, 42.56%; H, 4.64%; N, 24.78%.

Chromatographic analysis

The purity of obtained compounds **1** and **2** was examined using thin-layer chromatography (TLC). The stationary phase was TLC glass plates (5 × 10 cm RP-18F_{254s} (Merck, Darmstadt, Germany); the mobile phase was analytically pure chloroform, methanol and diethyl ether in the ratio 1.5:1.5:2. The calculated factors (rf) were rf = 0.53 for compound **1** and rf = 0.78 for compound **2**.

Measurement of nitric oxide generation

Sulfydryl agents – L-cysteine hydrochloride monohydrate (LC) and L-cysteine ethyl ester hydrochloride (LCEE), were purchased from Sigma-Aldrich. NO was generated from the interaction between compounds **1** or **2** and LC or LCEE in phosphate buffer (50 mM, pH 7.7).

Gaseous NO released as a result of reaction with thiols was measured using a polarographic method using an ISO-NO sensor selective for NO and an ISO-NO isolated nitric oxide meter (World Precision Instruments, Inc. Sarasota, FL, USA). The measurements were recorded and the results analysed using a specialist software package Duo-18 (World Precision Instruments, Inc. Sarasota, FL, USA) designed for electrochemical measurements associated with the release of NO. One measurement per minute was recorded.

All the determinations were carried out in aqueous solutions of the same volume (10 mL) at 37°C under anaerobic

conditions in argon atmosphere, with automatic stirring of the solution. The measuring electrode was introduced into the solution to a depth of 5 mm.

Calibration of the method involved synchronization of the readings from the electrode (pA) embedded in water and then in 1 M NaCl. Sensor calibration was carried out using the method of chemical generation of NO in the reaction of potassium nitrite with potassium iodide in acid medium. The sensor was embedded in a solution containing 0.1 M H_2SO_4 and 0.1 M KI, supplemented consecutively with 50 μL, 100 μL, 150 μL and 200 μL 50 μM potassium nitrite. The quantity of NO generated in the solution corresponded with the quantity of potassium nitrite added. It was calculated (in nmol) on the basis of the following equation: $M_1 \times V_1 = M_2 \times V_2$ (where M_1 = initial concentration; V_1 = initial volume; M_2 = final concentration; V_2 = final volume). A curve illustrating the dependence of current intensity changes (pA) on changes in NO concentration (nmol) was drawn. The slope of the curve was 4.62 pA $nmol^{-1}$ NO, and the linear correlation coefficient was r = 0.99873.

Nitric oxide generation

A selective isolated NO (ISO-NO) sensor connected to an ISO-NO meter was placed in a solution containing 1.5 mmol LC or LCEE in phosphate buffer pH 7.7, heated to 37°C, through which oxygen-free argon was passed for 15 min. The solution containing 0.5 mmol of the investigated nitrate (in phosphate buffer pH 7.7, heated to 37°C and oxygen-free as a result of saturation with argon) was added. The solutions were mixed with a magnetic stirrer at 37°C and the NO generated was measured (in μmol) after 1 minute. Five separate determinations were carried out for each investigated nitrate and the mean ± s.e.m. calculated (Table 1).

Pharmacological studies

The study was conducted on male SH rats weighing 350–400 g. The animals had free access to standard feed and tap water. Rats were housed in standard plastic cages, 10 animals per cage, at a constant temperature of 21 ± 1°C in a 12 h light–dark cycle. Animals were acclimatized to the environment and equipment for 3 weeks before the study. Preliminary examinations were carried out in the second and third week of acclimatization, in order to select appropriate animals: rats with wide fluctuations in BP were excluded from the study. The experiments were conducted between 0800 and 1600.

Table 1 Nitric oxide (NO) generation from compounds **1** and **2** with the thiols L-cysteine hydrochloride monohydrate (LC) and L-cysteine ethyl ester hydrochloride (LCEE)

Compound	Thiol	NO generation (μmol min^{-1})
1	LC	0.0112 ± 0.0005
1	LCEE	0.0097 ± 0.0006
2	LC	0.0182 ± 0.0003
2	LCEE	0.0124 ± 0.0007

Data are mean ± s.e.m. from five separate experiments.

All experimental procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals and were approved by the Local Ethics Committee for the Experiments on Animals (no. LKE/32/2005).

Single-dose studies

Rats with hypertension of about 200/130 mmHg were divided into 10 groups of six animals. The tested compounds were suspended in 1% methylcellulose solution in water, and administered intraperitoneally (i.p.): **1a** and **2a** at 1 mL kg^{-1}; **1** and **2** at 0.2 mL kg^{-1}. Doses of the compounds were as follows: dyphylline **1a**, 50 and 100 mg kg^{-1}; dyphylline nitrate **1**, 25, 59 and 118 mg kg^{-1}; proxyphylline **2a**, 25 and 50 mg kg^{-1}; proxyphylline nitrate **2**, 10, 30 and 60 mg kg^{-1}. Control rats received the same volume of the drug vehicle (1% methylcellulose).

Multiple administration of the compounds

Rats used in long-term studies were divided into four groups of animals. Doses of the tested compounds were chosen on the basis of results from the single-dose experiments: **1a,** 50 mg kg^{-1}; **1**, at an equivalent dose of 60 mg kg^{-1}; **2a,** 50 mg kg^{-1}: **2,** 59 mg kg^{-1}. The rats received i.p. injections of the test compounds twice daily at 8 h intervals for 9 days. Then, following a 16 h wash-out period, an additional single dose of the compound was given i.p.

Arterial BP measurement

Arterial BP was measured in conscious rats using a manometer (Letica, Panlab S.L., Barcelona, Spain), using the tail-cuff method described by Górska & Andrzejczak (2003). Before the measurements, to calm the animals and dilate the tail blood vessels, the rats were placed in a warming chamber (about 34°C) for 30 min. Arterial BP (systolic, diastolic and average) was measured before drug administration and 30 and 60 min after the injection. In rats treated with multiple doses of the tested compounds, arterial BP measurements were conducted on the 10th day of treatment. At least three measurements were carried out at each time point.

Statistical analysis

Results are expressed as mean ± s.e.m. The normality of distribution was checked by means of the Kolmogorow–Smirnoff test with Lilliefor's test. The statistical evaluation was performed using analysis of variance (ANOVA); post-hoc comparisons were performed by means of least significant differences test. If data were not normally distributed, statistical evaluation was performed using ANOVA (Kruskall–Wallis) and the Mann–Whitney and Wilcoxon tests. Differences were considered significant at $P < 0.05$. Initial values of arterial BP were taken as 100%.

Results and Discussion

Chemical and analytical results

New nitrate analogues of 7-(2,3-dihydroxypropyl)theophylline (**1a**) and 7-(2-hydroxypropyl)theophylline (**2a**) were synthesized:

7-(2-hydroxy-3-nitrooxypropyl)theophylline (**1**) and 7-(2-nitrooxypropyl)theophylline (**2**). Because of the presence of an ONO_2 group in their structure, these compounds were expected to be NO donors, a property which could increase their vasodilating and hypotensive actions.

Structures of compounds **1** and **2** were confirmed by spectroscopic methods and elemental analysis. The IR spectra of compounds **1** and **2** contain strong signals of asymmetric oscillations and weaker signals of symmetric ones of the ONO_2 group, as well as the signals of C=O and OH groups at appropriate frequencies (ν, cm^{-1}). ^{1}H-NMR spectra contain the signals of all protons present in the structures of both compounds at appropriate ppm values (Figure 1). The obtained compounds **1** and **2** were pure, confirmed by TLC.

Compounds **1** and **2** were subjected to in-vitro tests with a polarographic method using an ISO-NO sensor selective for NO. The reaction of compounds **1** and **2** with the sulfydryl compounds LC and LCEE was used as a model corresponding to the metabolism of therapeutic organic nitrates. Release of NO as a result of incubation of the reagents in phosphate buffer (pH 7.7, 50 mM) at 37°C under anaerobic conditions was observed. The quantity of NO released was measured using a selective ISO-NO sensor (Xian et al 2000).

The effects of parameters such as reaction temperature, pH and the ratio of nitrate and thiol quantities on the amount of generated NO were examined in preliminary experiments. A temperature of 37°C, pH 7.7 and thiol-to-nitrate ratio of 3:1 were chosen as the optimum reaction parameters for further measurements. We also found that stereospecificity of thiols used in the experiments had no effect on the quantity of NO generated.

NO was not generated spontaneously by compounds **1** and **2** in the absence of a cofactor. Of the thiols used, LC generated the highest quantities of NO from nitrates **1** and **2**. The activity of LCEE was markedly lower in comparison with that of LC. Compound **2** released more NO than compound **1** with both LC and LCEE (Table 1).

The synthesis of 7-(hydroxyalkyl)theophylline nitrates, involving esterification of 7-(2,3-dihydroxypropyl)theophylline **1a** and 7-(2-hydroxypropyl)theophylline **2a** with fuming nitric acid was performed. The methodology of these reactions was developed experimentally, and each of the reactions had a different course. Substrate **1a** contains two alcohol groups differing in bond orders, which undergo nucleophilic substitution according to two different mechanisms. The primary alcohol group reacts according to the S_N2 mechanism involving bimolecular nucleophilic substitution, whereas the secondary group is substituted with a single molecule according to the S_N1 mechanism. The differences in reaction mechanisms of the two alcohol groups present in the structure of compound **1a** made it possible, by applying appropriate reaction conditions, to obtain a mononitrooxyl derivative **1**. The reaction of **1a** and **2a** esterification with fuming nitric acid had to be conducted at a precisely adjusted temperature. Too low a temperature caused an excessive deceleration of the reaction, whereas too high a temperature led to partial decomposition of the products. In addition, the reaction mixture had to be completely homogeneous, which was achieved by comminution of the reagents and vigorous stirring.

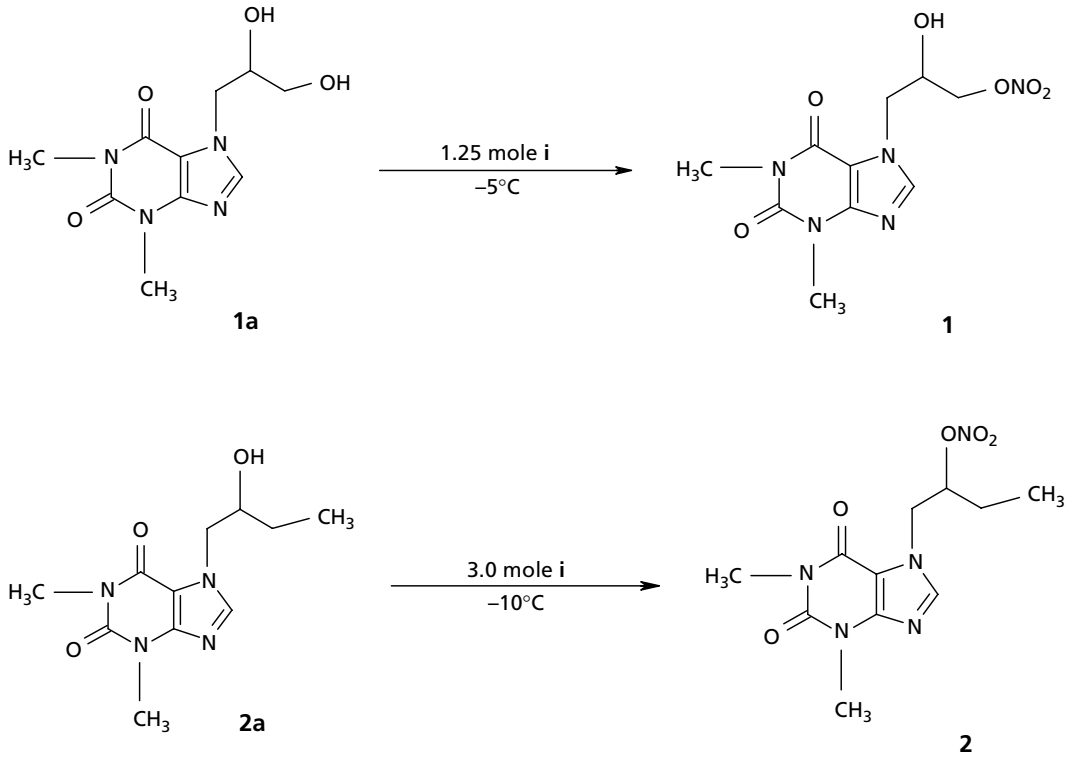

Figure 1 Synthesis of compounds **1** and **2**. Reagent **i** is fuming nitric acid.

The results of the experiments demonstrate that NO can be generated in-vitro by organic nitrate derivatives of theophylline as a result of reactions of compounds **1** and **2** with sulfydryl compounds LC and LCEE. Under the experimental conditions used, LC generated higher quantities of NO from compounds **1** and **2** than LCEE. This is consistent with our previous observations of NO generation by another group of nitrates in reactions with the same thiols (Korzycka 2002; Korzycka & Górska 2005), as well as with reports by other authors suggesting that cysteine may play a crucial role in the metabolism of organic nitrates in living organisms (Feelisch & Noack 1987; Chong & Fung 1991).

Our experiments show that NO generation can be quantified using a polarographic method with a selective ISO-NO sensor connected to an ISO-NO isolated NO meter. Compound **2** generated more NO than compound **1** in reactions with both thiols. This may result from the more lipophilic properties of compound **2** (log *P* 4.85) compared with compound **1** (log *P* 4.10).

In preliminary tests carried out to establish the optimum parameters for analysis of NO generation, a linear correlation between the quantity of NO generated and thiol concentration was observed. However, higher concentrations of sulfydryl compounds could not be used in the experiments because of a decrease of their solubility in the reaction medium at higher concentrations. The rate of reaction between organic nitrates **1** and **2** and thiols LC and LCEE is largely dependent on temperature, and for that reason the reaction mixture had to be carefully regulated in each test to obtain comparable results.

Pharmacological results

Effects of a single doses of the test compounds on BP

The effects of a single dose (100 mg kg^{-1}) of dyphylline **1a** and its nitrate **1** on arterial BP in SH rats are shown in Table 2. When compared with vehicle-treated control animals, a single dose of compound **1a** resulted in a significant increase in systolic and mean BP after 30 min, followed by a decrease in diastolic and mean BP. A single dose of compound **1** (118 mg kg^{-1}) significantly decreased systolic, diastolic and mean BP at the two time points examined compared with the group receiving **1a** (except diastolic BP at 60 min) and the control rats. Derivative **1** produced a significantly greater decrease in the mean BP than compound **1a** ($P = 0.000041$, $P = 0.002531$ at 30 and 60 min, respectively).

Dyphylline **1a** (50 mg kg^{-1}) significantly decreased all BP parameters 30 min after injection, and diastolic BP after 60 min compared with the control group. A single dose of compound **1** (at an equivalent dose) produced a statistically significant decrease in systolic, diastolic and mean BP at both 30 and 60 min. The hypotensive effect of compound **1** on mean BP was higher than with **1a** ($P = 0.000012$; $P = 0.000472$ at 30 and 60 min, respectively).

Compound **1** at the lowest tested dose caused a significant decrease in systolic, diastolic and mean BP 30 and 60 min after injection (except for systolic BP at 60 min) compared with the control group (Table 2).

The effects of a single dose of proxyphylline **2a** or proxyphylline nitrate **2** on arterial BP in SH rats are shown in Table 3. Although proxyphylline nitrate **2** (10 mg kg^{-1}) had a hypotensive effect, it did not significantly decrease systolic and mean BP at 60 min or diastolic BP 30 min after the injection. Compound **2a** (25 mg kg^{-1}) produced a significant decrease in diastolic BP 30 and 60 min after the injection, and in systolic and mean BP after 30 min. The nitrate ester **2**, at an equivalent dose, decreased all BP parameters after 30 and 60 min. The hypotensive effect of compound **2** was significantly greater than that of compound **2a** at 60 min. A single dose of **2a** (50 mg kg^{-1}) and **2** (60 mg kg^{-1}) produced similar decreases in BP during the observation period.

Effects of long-term administration of test compounds on BP

Mean values of arterial BP in SH rats treated with xanthines **1a, 1, 2a** and **2** twice daily for 9 days and recorded 16 h after injection of the 18th dose are given in Table 4. All four compounds produced significant decreases in the BP parameters. The hypotensive effect of dyphylline nitrate **1** was similar to that of dyphylline **1a** (mean BP 80.6 ± 2.5% and 82.2 ± 1.6% of the initial values, respectively). Proxyphylline **2a** caused significantly greater decreases in diastolic and mean BP than did proxyphylline nitrate **2**. Of the xanthines studied, compound **2a** had the greatest effects on diastolic and mean BP.

The effects of a single i.p. injection of the parent compounds **1a** and **2a**, and nitrate analogues **1** and **2**, on BP in SH rats pretreated for 9 days with the same compound are presented in Table 5.

In these pretreated rats, a single i.p. injection of compound **1a** produced a statistically significant increase in systolic BP from the baseline value 30 min after the injection, whereas compound **1a** produced a statistically significant decrease in systolic BP after 30 and 60 min. Compound **2a** and its derivative **2** significantly decreased systolic BP after 60 and 30 min, respectively. Proxyphylline nitrate had a more marked effect on systolic BP at 30 min than the parent compound and other tested xanthines.

The experiments assessing pharmacological activity of dyphylline and proxyphylline and new compounds **1** and **2** were carried out on SH rats, a commonly accepted animal model of hypertension. This strain of rats is characterized by an increase in cardiac noradrenaline release (Zugck et al 2003), renal retention of water and sodium (Trippodo et al 1981), kidney vascular resistance and a general increase in vascular resistance in almost all tissues (Evenwel et al 1983).

The haemodynamic changes observed in SH rats and the complex mechanism of action of xanthines have common features. The influence of theophylline on arterial BP is not unambiguous and depends on many factors, including the route of administration, plasma concentration of the drug, other drugs used and the condition of subjects participating in a study. Warren and coworkers (1983) studied the effect of posture on the sympathoadrenal response to intravenous theophylline in six normal subjects and found that the drug had little or no effect with the volunteers supine whereas when the subjects were standing theophylline caused tremor, a peak heart rate of 99 ± 6 beats min^{-1} and elevation of plasma cyclic AMP and cyclic GMP, adrenaline and noradrenaline concentrations. There was a small elevation in systolic BP with theophylline, both in subjects lying and standing, but this effect

Table 2 Effects of a single dose of dyphylline (**1a**) and dyphylline nitrate (**1**) on arterial blood pressure (BP) in spontaneously hypertensive rats 30 and 60 min after administration

	Systolic BP						**Diastolic BP**						**Mean BP**					
Compound	M	1	1a	1	1a	1	M	1	1a	1	1a	1	M	1	1a	1	1a	1
Dose ($mg\ kg^{-1}$)		25	50	59	100	118		25	50	59	100	118		25	50	59	100	118
30 min	99.0 ± 0.5	93.0 ± 1.6*[a]	79.0 ± 0.43*[a]	80.1 ± 1.2 *[a]	126.2 ± 2.5*[a]	78.1 ± 1.1*[ab]	101.1 ± 0.9	87.3 ± 0.5*[a]	90.5 ± 1.5*[a]	78.8 ± 0.9*[ab]	105.9 ± 2.9	86.3 ± 1.5*[ab]	99.3 ± 0.5	80.9 ± 0.8*[a]	85.2 ± 0.5*[a]	77.56 ± 0.6*[ab]	114.5 ± 2.8*[a]	82.6 ± 1.1*[ab]
60 min	99.8 ± 0.8	96.9 ± 1.1*	97.6 ± 1.1	80.6 ± 1.4*[ab]	95.3 ± 0.9[a]	78.0 ± 2.3*[ab]	103.8 ± 2.9	87.0 ± 0.6*[a]	97.4 ± 0.9[a]	92.9 ± 2.9[a]	89.1 ± 1.8*[a]	87.8 ± 1.4*[a]	101.8 ± 2.0	82.5 ± 0.6*[a]	97.2 ± 0.9	87.1 ± 1.7*[ab]	91.7 ± 1.3*[a]	83.3 ± 1.5*[ab]

Each parameter is expressed as percentage of the initial value and is the mean ± s.e.m. (n = 6); M, control rats, which received an i.p. injection of drug vehicle, methylcellulose.
*$P < 0.05$ vs 100% as initial value; [a]$P < 0.05$ vs control (M); [b]$P < 0.05$ vs parent compound (**1a**).

Table 3 Effects of a single dose of proxyphilline (**2a**) and proxyphilline nitrate (**2**) on arterial blood pressure (BP) in spontaneously hypertensive rats 30 and 60 min after administration

	Systolic BP						**Diastolic BP**						**Mean BP**					
Compound	M	2	2a	2	2a	2	M	2	2a	2	2a	2	M	2	2a	2	2a	2
Dose ($mg\ kg^{-1}$)		10	25	30	50	60		10	25	30	50	60		10	25	30	50	60
30 min	99.0 ± 0.5	87.6 ± 2.0*[a]	74.7 ± 1.4*[a]	74.9 ± 1.5*[a]	68.3 ± 2.5*[a]	67.3 ± 2.6*[a]	101.1 ± 0.9	91.1 ± 0.8	79.5 ± 5.3*[a]	74.7 ± 5.4*[a]	70.7 ± 5.3*[a]	71.7 ± 4.9*[a]	99.3 ± 0.5	89.4 ± 3.5*[a]	77.2 ± 3.3*[a]	74.8 ± 3.2*[a]	70.12 ± 8.61*[a]	70.1 ± 3.5*[a]
60 min	99.8 ± 0.8	101.8 ± 1.5	96.3 ± 1.1*	80.0 ± 0.9*[ab]	68.6 ± 3.4*[a]	69.3 ± 3.7*[a]	103.8 ± 2.9	94.3 ± 2.2[a]	95.8 ± 2.4[a]	66.7 ± 2.0*[ab]	70.4 ± 3.4*[a]	69.5 ± 3.4*[a]	101.8 ± 2.0	97.4 ± 1.1	95.9 ± 1.7	71.4 ± 1.4*[ab]	70.75 ± 7.88*[a]	70.7 ± 3.2*[a]

Each parameter is expressed as percentage of the initial value and is the mean ± s.e.m. (n = 6); M, control rats, which received an i.p. injection of drug vehicle, methylcellulose.
*$P < 0.05$ vs 100% as initial value; [a]$P <$ 0.05 vs control (M); [b]$P < 0.05$ vs parent compound (**2a**).

Table 4 Effects of prolonged administration (twice daily for 9 days) of dyphylline (**1a**, 50 mg kg^{-1}), dyphylline nitrate (**1**, 59 mg kg^{-1}), proxyphylline (**2a**, 50 mg kg^{-1}) and proxyphylline nitrate (**2**, 60 mg kg^{-1}) on arterial blood pressure (BP) in spontaneously hypertensive rats. Arterial BP was measured 16 h after the last injection

	1a	*P* value	1	*P* value	2a	*P* value	2	*P* value
Systolic	83.3±2.5	*0.001	80.5±3.0	*0.001	79.8±4.1	*0.003	79.3±1.8	*0.0003
Diastolic	81.7±2.4	*0.003, [a]0.0002	81.3±3.8	*0.003, [a]0.0003	58.9±4.8	*0.003	79.3±3.3	*0.0008, [a]0.0007
Mean	82.2±1.6	*0.00004, [a]0.0005	80.6±2.5	*0.003, [a]0.02	67.2±3.4	*0.00007	9.3±2.7	*0.0003, [a]0.004

Each parameter is expressed as a percentage of the initial value and represents the mean ± s.e.m. (n = 6);
*$P < 0.05$ vs 100% initial value; [a]$P < 0.05$ vs **2a**.

was not statistically significant. The authors suggested that theophylline may potentiate the increase in sympathetic activity that occurs on standing. The low level of stimulation of β_2-adrenoceptors, which occurs when subjects rest, did not appear to account for this amplification. De Galan and colleagues (2002) examined the effects of theophylline on hypoglycaemia in type 1 diabetes. Before hypoglycaemia, theophylline slightly increased heart rate (3.4 ± 0.8 beats min^{-1}) in control subjects, but did not affect BP in either diabetic patients or in control subjects. Theophylline significantly increased heart rate responses to hypoglycaemia in patients as well as in the control group, and decreased the systolic BP in diabetic patients. In the presence of theophylline, responses of diastolic BP, pulse pressure and heart rate in both groups, and systolic BP in diabetic patients, were elicited at significantly higher glucose level. Compared with healthy control subjects, theophylline largely normalized haemodynamic responses to hypoglycaemia in diabetic patients.

The doses of 100 mg kg^{-1} for compound **1a** and 50 mg kg^{-1} for **2a**, as well as appropriate equivalent doses of nitrates **1** and **2**, used in our studies were selected on the basis of a literature survey (Warszawski et al 1978; Selvig 1981; Blake et al 1988; Nadai et al 1992; Zeruesenay et al 1992; Bruguerolle & Dubus 1999; Park et al 1999; Onodera et al 2001). The doses were then reduced on the basis of the observed effect, with assessment of their efficacy. In clinical studies, Joos et al (1979) observed that proxyphylline administered in combination with dyphylline (in the preparation Neophyllin – each tablet contains 56 mg proxyphylline and 84 mg dyphylline) caused bronchodilation, which indicates the effectiveness of the above doses.

A single i.p. injection of the tested compounds produced a statistically significant decrease in BP. The hypotensive effect was observed following administration of each dose of dyphylline nitrate **1** (25, 59 and 118 mg kg^{-1}), proxyphylline **2a** (25 and 50 mg kg^{-1}), proxyphylline nitrate **2** (10, 30 and 60 mg kg^{-1}) and dyphylline **1a** (50 mg kg^{-1}). Compound **1a** at 100 mg kg^{-1} caused a transient increase in arterial BP, which was probably caused by a reflex tachycardia due to considerable reduction in BP resulting from the use of too high a dose. We propose a more reliable explanation of the hypertensive effect of compound **1**. Dyphylline, at a higher dose, could release catecholamines; this effect was described as one of the mechanisms of action of xanthines (Barnes 2003). Therefore, stimulation of the adrenergic system could dismantle the hypotensive effect observed after the administration of another dose. In the case of dyphylline nitrate, used in a dose equivalent to that of dyphylline, release of NO could counterbalance the action of noradrenaline/adrenaline on the arterial pressure, finally resulting in a significant decrease in the BP parameters measured. It appeared that in SH rats, single doses of dyphylline nitrate produced a more pronounced hypotensive effect than dyphylline. This effect could result from the presence of the NO group, and may suggest an ability of compound **1** to generate the radical in-vivo. In line with this assumption, compound **1** was able to release NO in-vitro. Hypotensive actions exerted by compound **1** at doses of 59 and 118 mg kg^{-1} did not differ significantly, suggesting saturation of the effect of ester **1** (i.e. maximum utilization of the NO release mechanisms).

Proxyphylline nitrate **2** and its parent compound **2a**, given acutely, produced comparable hypotensive effects, with an exception of changes observed 60 min after the injection, when the decreases in diastolic and systolic BP produced by 60 mg kg^{-1} of compound **2** were higher than those produced by the equivalent dose (50 mg kg^{-1}) of compound **2a**. Compound **2** at the lowest tested dose slightly but significantly reduced systolic and mean arterial pressure only at 30 min. This hypotensive effect was significantly less than that produced by compound **2a** at 25 mg kg^{-1}. Taking into account the above results, it seems likely that the hypotensive action of ester **2** is not related to its activity as a NO donor.

All the tested xanthines given twice daily to SH rats for 9 days significantly reduced BP; this effect persisted for at least 16 h after the last dose of the drug. The greatest decrease in diastolic and mean BP was observed after prolonged treatment with proxyphylline. Nitrates **1, 2** and dyphylline exhibited comparable hypotensive activities, an observation indicating that the haemodynamic effect under consideration depended on the common properties of xanthines rather than on release of the nitrate group. Furthermore, it appears that the hypotensive effect of the tested compounds given to SH rats at repeated doses was not associated with PDE inhibition or catecholamine release, as we did not observe an increase in arterial BP. Increase in plasma adrenaline and noradrenaline concentrations during stress and acute asthma attack (Ind et al 1985) resemble intensified sympathetic activity in SH rats. Different effects were observed by Whyte and colleagues (1988), who examined the effects of infusion of salbutamol alone or in conjunction with theophylline, adrenaline, or a combination of these two drugs (as in acute asthma), on heart rate and BP in a single-blind study in healthy volunteers. The β_2-agonist infusion produced a fall in diastolic BP and a rise in systolic BP. These changes were not altered by any of the drug combinations tested.

Table 5 Effect of a single i.p. dose of dyphylline (**1a**, 50 mg kg^{-1}), dyphylline nitrate (**1**, 59 mg kg^{-1}), proxyphilline (**2a**, 50 mg kg^{-1}) and proxyphilline nitrate (**2**, 60 mg kg^{-1}) on arterial blood pressure (BP) at 30 min and 60 min, to spontaneously hypertensive rats pretreated with the same compound twice daily for 9 days

	Systolic BP				Diastolic BP				Mean BP			
	1a	**1**	**2a**	**2**	**1a**	**1**	**2a**	**2**	**1a**	**1**	**2a**	**2**
30 min	104.0 ± 1.6 (*P = 0.046; [a]P = 0.000036)	96.9 ± 1.2 (*P = 0.0486; [a]P = 0.0196)	87.1 ± 5.7 ([a]P = 0.0196)	70.6 ± 7.1 (*P = 0.00621)	104.3 ± 8.8	99.5 ± 2.5	90.5 ± 8.1	89.2 ± 8.4	99.3 ± 3.6	98.7 ± 1.7	88.7 ± 6.7	87.7 ± 7.1
60 min	98.8 ± 1.5	94.5 ± 1.3 (*P = 0.0048)	78.5 ± 7.0 (*P = 0.02215)	89.0 ± 5.1	97.0 ± 3.6	100.7 ± 4.07	103.6 ± 9.0	97.6 ± 8.8	97.3 ± 2.1	98.15 ± 2.5	90.9 ± 7.7	93.1 ± 6.9

Each parameter is expressed as a percentage of the initial value and represents the mean ± s.e.m. (n = 6); *P < 0.05 vs 100% initial value; [a]P < 0.05 vs **2a**.

We examined the effect of a single dose of proxyphylline, dyphylline and their nitrate derivatives on arterial BP 30 min and 60 min after administration to SH rats that had been pretreated with the respective compound for 9 days. This additional dose of nitrates **1** and **2** markedly decreased systolic BP. Proxyphylline nitrate had the strongest hypotensive effect of the xanthines. None of the compounds affected diastolic or mean BP. Flatt et al (1989) have reported that theophylline-induced changes in diastolic BP did not differ from those produced by this xanthine given together with fenoterol. Fenoterol at doses of 600 μg and 800 μg produced significantly greater increases in systolic BP in patients treated with theophylline (for 1 week) than in the control group. The authors suggested that long-term treatment with oral theophylline (in order to achieve steady-state serum drug concentrations) does not have discernible circulatory effects, a phenomenon that probably reflects development of tolerance to the cardiovascular effects of this drug.

Conclusion

The tested xanthines, given both acutely or in repeated doses, exerted hypotensive effects in SH rats. Insertion of a nitrate group into the xanthine structure did not markedly modify the potential of these compounds to reduce arterial BP.

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