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Original article

NO-donors, part 9 [1]: diazeniumdiolates inhibit human platelet aggregation and induce a transient vasodilatation of porcine pulmonary arteries in accordance with the NO-releasing rates

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Abstract

Diazeniumdiolates (NONOates), among them a ciprofloxacin-diazeniumdiolate hybrid compound, were synthesized and the pH-, temperature- and structure-dependent liberation of nitric oxide (NO) was monitored by laser magnetic resonance spectroscopy (LMRS). The compounds induced a transient and reversible relaxation (EC_{50} 8.3–150 nM) of pulmonary arteries independently from intact endothelium by stimulation of guanylyl cyclase (sGC). Increase in vascular cGMP was observed and blocking sGC with ODQ, an inhibitor of the NO-sensitive guanylyl cyclase, induced a rightward shift of the concentration–response curves. Repeated exposure did not show homologous desensitization. ADP-induced platelet aggregation (IC_{50} = 0.15–3 μM, IC_{50} for SNP: 2 μM) and collagen-induced aggregation were potently inhibited. Preincubation with ODQ also diminished these inhibitory effects.

Keywords: Diazeniumdiolates; Nitric oxide; Laser magnetic resonance; Vasodilatation; Platelet aggregation inhibition; Ciprofloxacin

1. Introduction

Nitric oxide (NO) is an important regulator of physiological cellular processes including vascular tone, platelet function, neurotransmission and cell proliferation. A number of disorders are associated with impaired synthesis and/or increased degradation of vascular NO, such as hypertension, hypercholesterolaemia, endothelial dysfunction and diabetes mellitus [2,3]. The reduced activity of vascular (endothelial) NO is also likely to play a significant role in the development of atherosclerosis. The suppression of the endothelium to produce biologically active NO could also predispose the vascular wall to platelet adhesion and constriction as consequence of liberation of vasoactive substances from damaged platelets. NO activates the soluble guanylyl cyclase (sGC) leading to an increase in cGMP that is believed to induce vasodilatation and inhibition of platelet aggregation.

Therefore, besides the increase in endothelial NO production via the receptor-mediated activation of eNO-synthase, the development of NO-releasing drugs (NO-donors) is of special interest [4,5]. The widely used nitrovasodilators such as glyceryl trinitrate (GTN) require biotransformation for liberation of NO and show tolerance when used therapeutically. Diazeniumdiolates (short name: NONOates) have been brought into the focus of interest as NO-releasing therapeutics due to their ability to release NO spontaneously under physiological conditions [6–8]. The inhibitory effect of NONOates on platelet aggregation and their antiproliferative action were demonstrated in several experimental studies [8–13]. Intrapericardial administration of long-lived diazeniumdiolates suppressed smooth muscle cell proliferation and thus reducing neointima formation after percutaneous transluminal coronary angioplasty in pigs [14]. NO-releasing

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diazeniumdiolate-coated polymers, which were used as vascular grafts in sheep, proved to be as thromboresistent prosthetic vascular implantation material [15]. One of the limiting factors of NO as platelet aggregation inhibitor is its short biological half-life.

Previously, we have demonstrated the vasorelaxant response of isolated porcine coronary arteries to NONOates [16]. In continuation of these studies, we now report on the synthesis of some diazeniumdiolates (Fig. 1), their profiles of NO-liberation and their effects on human platelet aggregation and vascular tone in isolated porcine pulmonary compared to SNP.

2. Chemistry

The diazeniumdiolates were synthesized by addition of nitric oxide to the appropriate secondary amines in anaerobic methanolic solutions of sodium or potassium methoxide in a stainless steel (SS) autoclave with a Teflon coated reaction cell, as we have described previously [6]. Generally the solid products were separated by suction, washed with ether and vacuum dried at room temperature, purification by recrystallization or chromatography is not possible due to decomposition. A downfield shift in the ^{1}H NMR spectra of the protons near to the diazeniumdiolate moiety indicates a successful transformation. Fig. 1 shows the general reaction of nitric oxide with the amines to produce the compounds **1**–**4**. Synthesis of **1** and crude **2** has been already performed by the group of Saavedra et al. [17,18]. When transforming *cis*,*trans*-3,5-dimethylpiperidine into the corresponding diazeniumdiolate **3**, the ratio of the *cis* and the *trans* isomer changed from 1:3 to 1:4. We prepared the ciprofloxacin derivative **4** to investigate any synergistic antimicrobial effects of the gyrase inhibitor and NO. The hybrid compound **4** showed the same but not a better antibacterial effect than ciprofloxacin against *E. coli*. The data will be reported together with other results later. Compound **4** could be obtained from the potassium salt of ciprofloxacin and 1 equiv. potassium methoxid or alternatively, using the acid and 2 equiv. $KOCH_3$. Synthesis afforded 3 days with 4 bar of NO, shorter reaction times yielded in mixtures of **4** and the starting material.

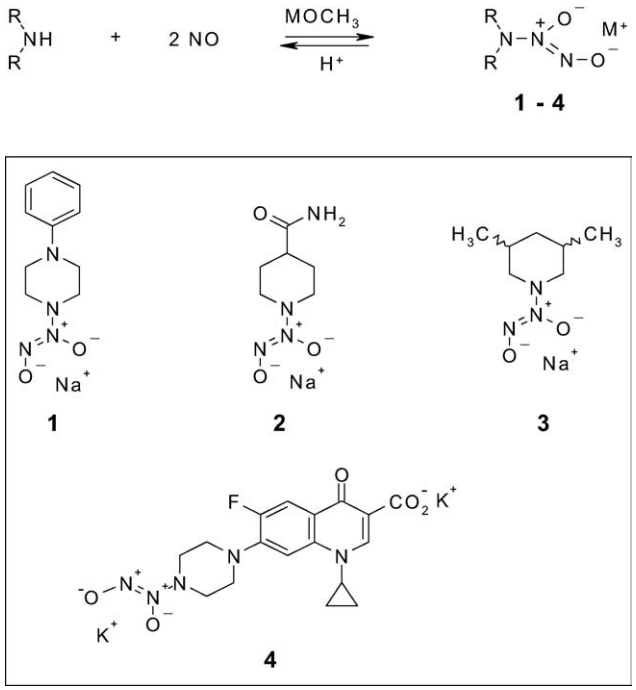

Fig. 1. Structures of the investigated diazeniumdiolates.

2.1. NO-liberation studies

We monitored the spontaneous NO-liberation of compounds **1**–**4** diazeniumdiolates in aqueous buffered solutions at pH 6.0, 7.4 and 8.0, created time-NO-liberation-profiles and determined the half-lives of our compounds following previously described methods [6]. The NO release was detected continuously in a flow through system using the Faraday-laser magnetic resonance spectroscopy (LMRS). LMR is a spectroscopic method for the study of paramagnetic molecules (radicals and ions) in the far-infrared and in the mid-infrared spectral region. By means of a magnetic field (Zeeman effect) the molecular transitions are tuned into resonance with the frequency of a line-tunable laser. The advantages of LMR are very high detection sensitivity and high spectral resolution. The sensitivity is achieved by polarization detection, one of the most sensitive spectroscopic methods in the mid-infrared wavelength range. For more details, data analysis see [6].

Fig. 2 shows the NO-liberation-profiles of compound **3** at pH 6.0, 7.4, and 8.0 at 20 °C. Varying the pH results in dramatic changes of the rate of NO generation. In our experimental setup the half-lives of the "fast releaser" **3** shifted from 4 to 198 s. Investigations of the other diazeniumdiolates demonstrated similar correlations between pH and NO-liberation. Table 1 gives the complete set of half-lives.

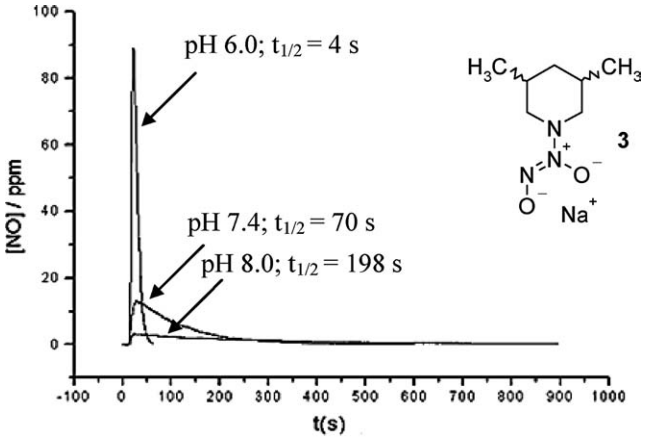

Fig. 2. Release of NO from the diazeniumdiolate **3**, monitored by LMRS.

Table 1
Relaxation periods (from maximum of relaxation to until complete recovery of the basal tone) induced by cumulative addition of the diazeniumdiolates to $PGF_{2\alpha}$-precontracted porcine pulmonary arteries compared to the NO-releasing half-lives of the diazeniumdiolates

Compounds	Half-life time (s) for NO release at			Duration of relaxation (min)
	pH 6.0	**pH 7.4**	pH 8.0	(mean ± S.E.M.)
3	4	**70**	198	23 ± 4 (n = 6)
2	13	**309**	877	27 ± 4 (n = 5)
1	42	**560**	1126	48 ± 3 (n = 4)
4	49	**609**	1313	51 ± 3 (n = 4)

3. Pharmacology and discussion

3.1. Relaxant responses of pulmonary arteries to the diazeniumdiolates

In porcine pulmonary arteries with intact endothelium $PGF_{2\alpha}$ (3 μM) caused a contractile response of 2.21 ± 0.1 g (n = 35). Bradykinin (10 nM) relaxed the precontracted arteries by 88.6 ± 2.2%. The addition of diazeniumdiolates to $PGF_{2\alpha}$-precontracted vascular rings resulted in concentration-dependent transient and reversible relaxation. The concentration–response curves for the relaxant effects are shown in Fig. 3. The most potent compounds **2** and **3** elicited relaxation already at 1 nM and maximum effects at 100–300 nM. Considering the EC_{50} values, SNP was significantly weaker effective than the compounds tested, but GTN was equipotent to **1** and **4**.

The relaxation induced by the diazeniumdiolates was found to be endothelium-independent, there were no significant differences in the relaxants effects between vessels with intact endothelium and mechanically endothelium-denuded vessels (Fig. 4). In order to confirm that the diazeniumdiolate-induced relaxation was associated with an increase in cGMP concentration due to the NO-mediated activation of the sGC, the arterial rings were preincubated with the NO-sensitive guanylyl cyclase inhibitor ODQ (10 μM) for 30 min. ODQ alone had no effect on vascular tone. In the presence of ODQ the concentration–response curves for the NONOates were shifted to the right in a parallel manner by about two orders of magnitude, as demonstrated for compound **2** in Fig. 4. The cGMP-level in the $PGF_{2\alpha}$-precontracted vascular rings (control) was significantly reduced when the vessels were preincubated with L-NAME, which inhibits the eNOS. An increase in cGMP was measured after pretreatment of the vessels with SNP, bradykinin and—very significantly—compound **2** (Fig. 5).

To prove whether desensitization of relaxation occurred after repeated application of NONOates, the rings were exposed to second challenge with diazeniumdiolates at the same submaximal concentration without an intermediate washout. The second relaxant response was not significantly reduced. Fig. 6 shows that after repeated bolus administrations of a diazeniumdiolate the subsequent relaxation is a transient and fully reversible one. When exposure to PGF2α-precontracted vessels to the diazeniumdiolates was repeated

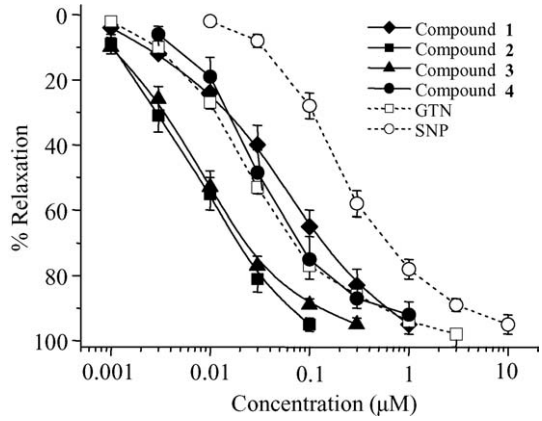

Fig. 3. Concentration–response relationship for the relaxation of $PGF_{2\alpha}$-precontracted pulmonary arteries induced by the diazeniumdiolates **1**–**4**, SNP and GTN. Mean ± S.E.M., n = 4–6.

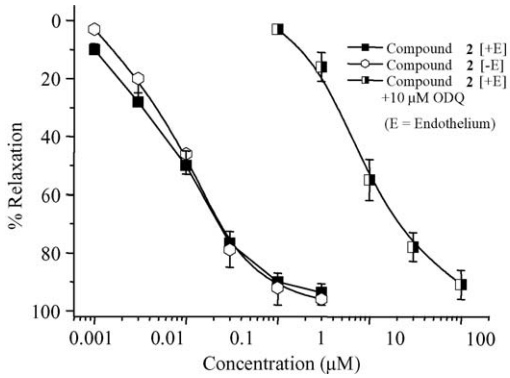

Fig. 4. Inhibition of compound **2**-induced relaxation of $PGF_{2\alpha}$-precontracted pulmonary arteries by preincubation with the guanylyl cyclase inhibitor ODQ. Mean ± S.E.M., n = 4.

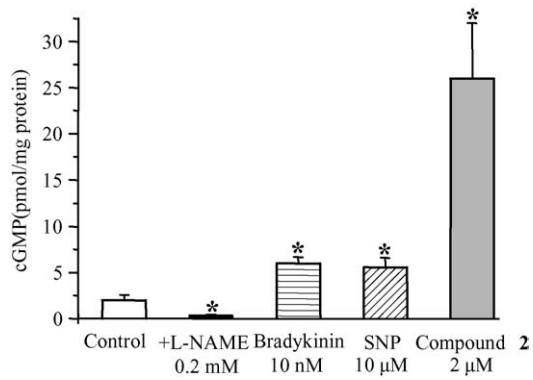

Fig. 5. Changes in cyclic GMP concentrations in $PGF_{2\alpha}$-precontracted porcine pulmonary arteries without (control) and after treatment with L-NAME, bradykinin, SNP and compound **2**, respectively. Mean ± S.E.M., n = 4. *) $P < 0.05$ compared with the control.

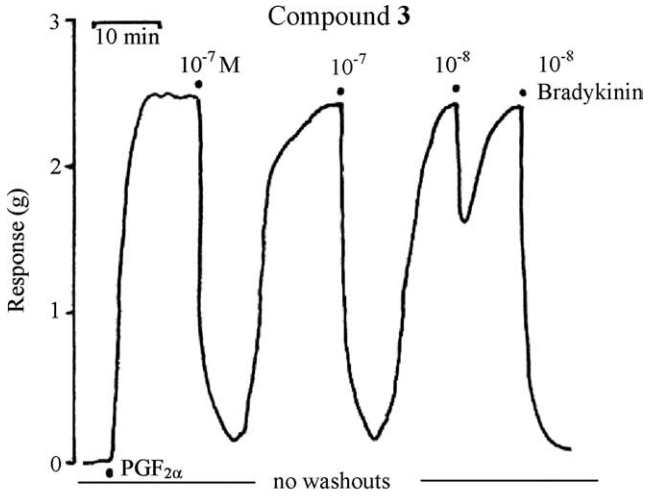

Fig. 6. Repeated relaxation of a $PGF_{2\alpha}$-precontracted porcine pulmonary artery by bolus administrations of compound 3 after recovery of the basal tone.

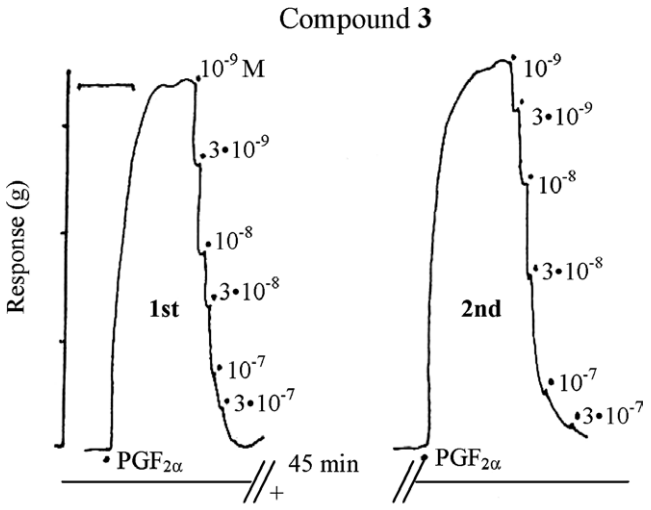

Fig. 7. Repeated relaxation of a $PGF_{2\alpha}$-precontracted porcine pulmonary artery by compound **3** after 45 min including washouts.

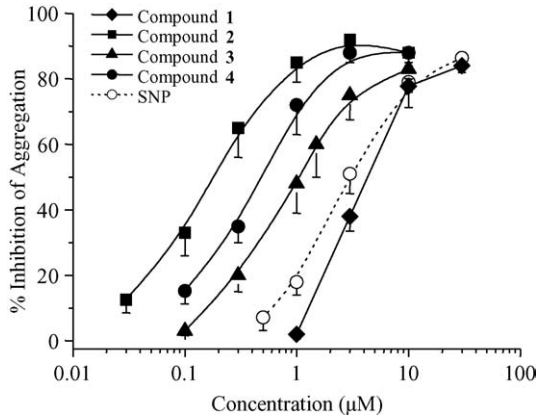

Fig. 9. Inhibition of ADP (5 μM)-induced human platelet aggregation in citrated plasma by compounds **1**–**4** and SNP. Mean ± S.E.M., n = 4–8.

after an interval of 45 min and three washouts, the compounds exhibited the same relaxant effect as observed after the first exposure (both experiments demonstrated for compound **3** in Figs. 6 and 7).

We also found that the duration of the relaxation period (min) from the maximum of the relaxation until the complete recovery of the vascular tone depends on the structure of the diazeniumdiolate and obviously correlates with the time-NO-releasing profiles of the NO-donors. Fig. 8 compares the slow releaser **1** with the faster **2**, all the correlations can be taken from Table 1.

3.2. Inhibition of platelet aggregation

NO-donors proved to be potent inhibitors of platelet aggregation [12,19]. The present studies confirmed that the diazeniumdiolates tested inhibited the ADP-induced platelet aggregation in human citrated plasma by 80–90% at maximum (Fig. 9). The concentrations required for significant inhibition were more than one order of potency higher than those for vascular relaxation, which is in accordance to the results published by Homer and Wanstall [19]. The compounds such as **4** inhibited also the collagen-induced platelet aggregation

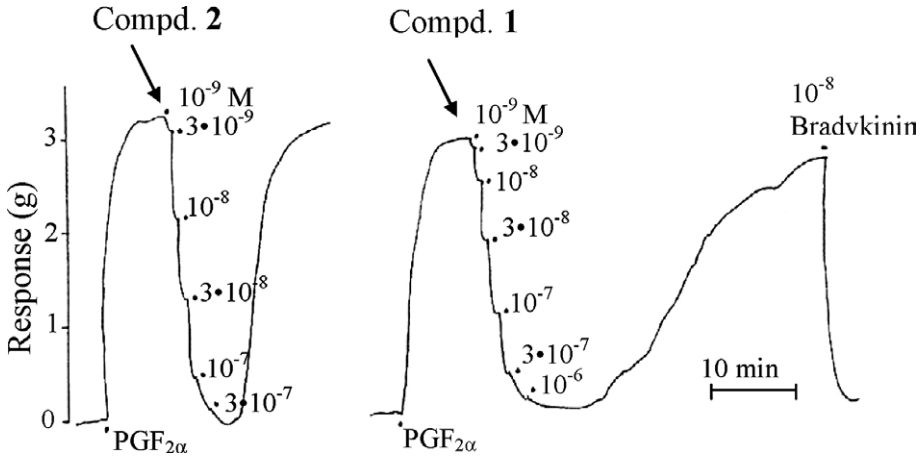

Fig. 8. Transient and reversible relaxations of $PGF_{2\alpha}$-precontracted porcine pulmonary arteries by a fast (compound **2**) and a slow (compound **1**) NO-releasing diazeniumdiolate.

in the same concentration range. When the platelet-rich plasma (PRP) was preincubated with ODQ (25 μM) for min, the inhibitory effect of the diazeniumdiolates was significantly diminished, but less potently as observed in the relaxation studies. Prolongation of the preincubation time from 2 to 15–30 min did not enhance or reduce significantly the inhibitory potency.

To demonstrate whether the compounds were able to inhibit platelet aggregation in whole blood, human citrated blood was pretreated with compound **2** (10 μM) or SNP (2 and 10 μM), respectively, at room temperature for 10 min. After centrifugation PRP was obtained and applied for platelet aggregation. The ADP-induced aggregation was inhibited by **2** by 44 ± 4% and by SNP 2 μM by 41 ± 4% and 10 μM by 73 ± 5%, respectively. Thus, inhibition of platelet aggregation might also be expected in whole blood.

It can be summarized that the diazeniumdiolates studied proved to be potent inhibitors of platelet aggregation (>SNP). They also elicit a strong endothelium-independent vasodilatation. There is no homologous desensitization of the vascular responses. The mechanism of action is certainly due to the direct release of NO from the compounds, which stimulates the sGC and thus producing cGMP in the vessels and platelets. This stands in contrast to vasodilators such as organic nitrates which need enzymatic [20] and non-enzymatic bioactivation steps to yield NO or other vasodilatating species such as thionitrates or thionitrites.

4. Experimental protocols

4.1. Chemistry

NO was purchased from Air liquide (France). NMR spectra were recorded on Bruker WM 500 (500 MHz) using D_2O as solvent and tetramethylsilan as internal standard (chemical shifts in δ, ppm).

*4.1.1. General procedure for preparation of the diazeniumdiolates **1–4***

An equimolar mixture of the secondary amine and sodium or potassium methoxide in methanol was transferred into an autoclave. The instrument used in preparation of diazeniumdiolates has been described in a previous paper [6]. The system was evacuated and rinsed with nitrogen three times, then charged with NO gas at **4** bar and kept under pressure for the time given below. The separated white precipitate was filtered and dried under vacuum or the solution was reduced under vacuum, cooled and a small amount of ether was added to induce precipitation.

*4.1.2. Sodium 1-(4-phenylpiperazin-1-yl)diazen-1-ium-1,2-diolate (**1**)*

From 1-phenylpiperazine (8.1 g, 0.05 mol) and sodium methoxide (2.7 g, 0.05 mol) in 100 ml methanol, reaction time 48 h at 4 bar NO. The product was filtered and dried i. vac. at room temperature yielding 5.5 g (45%) of a white solid; m.p. 220–222 °C ([17], 215–216 °C); ^{1}H NMR (0.1 M NaOD in D_2O): δ = 3.34 (m, 4H, piperazine), 3.40 (m, 4H, piperazine), 7.09 (t, J = 7.0 Hz, 1H, phenyl-4), 7.15 (d, J = 8.0 Hz, 2H, phenyl-3,5), 7.42 (dd, J = 8.0 Hz, 2H, phenyl-2,6).

*4.1.3. Sodium 1-(4-carboxamido-1-piperidyl)diazen-1-ium-1,2-diolate (**2**)*

From piperidine-4-carboxamide (2.56 g, 0.02 mol) and sodium methoxide (1.08 g, 0.02 mol) in 100 ml methanol, reaction time 48 h at 4 bar NO. The product was filtered and dried i. vac. at room temperature yielding 2.5 g (60%) of a white solid; m.p. 218–220 °C; ^{1}H NMR (0.1 M NaOD in D_2O): δ = 1.86 (m, 2H, H-3,5), 2.05 (m, 2H, H-3′,5′), 2.45 (m, 1H, H-4), 3.08 (m, 2H, H-2,6), 3.24 (m, 2H, H-2′,6′).

*4.1.4. Sodium cis/trans-1-(3,5-dimethyl-1-piperidyl)diazen-1-ium-1,2-diolate (**3**)*

From *cis*,*trans*-3,5-dimethylpiperidine (4.53 g, 0.04 mol) and sodium methoxide (2.8 g, 0.04 mol) in 50 ml methanol, reaction time 48 h at 4 bar NO. The gelatinous product was filtered and dried i. vac. at room temperature yielding 5.4 g (64%) of a white hygroscopic solid; m.p. > 230 °C; ^{1}H NMR (0.1 M NaOD in D_2O) of the major *trans*-isomer: δ = 0.68 (q, 1H, J = 12 Hz, H-4), 0.94 (d, J = 7 Hz, 6H, $2CH_3$), 1.76 (m, 1H, H-4′), 1.81–1.87 (m, 2H, H-3,5), 2.50–2.60 (t, J = 11 Hz, 2H, H-2,6), 3.06 (m, 2H, H-2′,6′). Minor isomer: δ = 1.03 (d, J = 7 Hz, 1.85H, $2CH_3$), 1.36 (t, J = 6 Hz, 0.62H, H-4,4′), 2.12 (m, 0.60 H, H-3,5), 2.74 (m, 0.61H, H-2,6), 3.06 (m, 0.6H, H-2′,6′). ^{13}C NMR (0.1 M NaOD in D_2O) of the major *trans*-isomer: δ = 21.32 (2 × CH_3), 32.82 (C-3, C-5), 43.02 (C-4), 61.77 (C-2, C-6). Minor *cis*-isomer: δ = 20.85 (2 × CH_3), 29.52 (C-3, C-5), 39.88 (C-4), 61.63 (C-2, C-6).

*4.1.5. Dipotassium 1-[4-(1-cyclopropyl-3-carboxy-6-fluoro-4-oxo-1,4-dihydroquinolin-7-yl)piperazin-1-yl]diazen-1-ium-1,2-diolate (**4**, Cipro/NO)*

From ciprofloxacin potassium salt (3.69 g, 0.01 mol) and potassium methoxide (0.7 g, 0.01 mol) in 120 ml methanol, reaction time 72 h at 4 bar NO. The mixture was reduced i. vac. to half of the volume, refrigerated and the precipitate was filtered and dried i. vac. at room temperature yielding 2.34 g (50%) of a white solid; m.p. > 230 °C; ^{1}H NMR (0.1 M NaOD in D_2O) δ = 1.05 (m, 2H, cyclopropyl), 1.35 (m, 2H, cyclopropyl), 3.4–3.5 (m, 9H, piperazine + cyclpropyl (1H)), 7.4 (d, J = 1 Hz,1H, H-8), 7.7 (dd, J = 1, 5 Hz, 1H, H-5), 8.5 (s, 1H, H-2); ^{13}C NMR (0.1 M NaOD in D_2O) δ = 10.26 (cyclopropyl-2), 37.56 (cyclopropyl-1), 51.79 and 54.11 (piperazine), 109.20 (C-8), 114.25 (d, $J_{c,F}$ = 22.75 Hz, C-5), 119.28 (C-3), 124.87 (d, $J_{c,F}$ = 7.5 Hz, C-4a), 141.11 (C-8a), 146.14 (d, $J_{c,F}$ = 11.01 Hz, C-7), 149.85 (C-2), 155.74 (d, $J_{c,F}$ = 245.75 Hz, C-6), 175.20 (C=O), 178.16 (C-4).

4.2. Biology

4.2.1. Substances

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) ADP, and bradykinin triacetate (Serva, Heidelberg, Germany); ODQ (1H [1,2,4]oxa-

diazolo[4,3-a]quinoxalin-1-one) (Alexis Corporation, Grünberg, Germany), Collagen (Nykomed, Munich, Germany); sodium nitroprusside (SNP), and GTN (Merck, Darmstadt, Germany), cGMP EIA Kit (Biomol, Hamburg, Germany).

4.2.2. Measurement of relaxant effects

Small branches of the pulmonary artery from pig lungs from the slaughter-house were carefully prepared as described previously [21,22]. Rings (2–3 mm in length) were placed in a 10 ml organ bath containing Krebs–Henseleit solution (mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11; pH 7.4; 37 °C) which was continuously gassed with a mixture of 5% CO_2 in O_2. A resting tension of 2 g was maintained throughout the experiments. Changes in tension were measured by isometric force transducers (Hugo Sachs Elektronic, March-Hugstetten, Germany). After an equilibration period of 60 min contractions were induced at 45 min intervals. The ring segments were initially contracted by addition of KCl (45 mM) and subsequently by PGF2α (3 μM) three times (at 45 min intervals) until the contractions had reached a constant value. Diazeniumdiolates were added to the organ bath at the plateau of contraction in cumulative manner or only single concentration. To establish cumulative concentration–response curves each successive agonist concentration was administered when the response had reached a plateau. ODQ was applied 10 min before the addition of the compounds. The diazeniumdiolate-induced relaxation was expressed as the percentage of PGF2α-induced contraction.

Endothelial cell function was assessed by the bradykinin (10 nM)-induced relaxation of PGF2α-precontracted ring segments. The relaxation was absent after mechanical removal of the endothelium which was achieved by gently rubbing the intimal surface of the rings with a small roughened plastic rod.

4.2.3. Determination of cGMP

When the relaxant effect in response to the diazeniumdiolates or bradykinin in $PGF_{2\alpha}$-precontracted pulmonary rings had reached the near-maximum level (usually after 90 s), the vessels were rapidly removed from the organ bath and frozen in liquid nitrogen [22]. The frozen samples were powdered by means of a dismembrator and then treated with 0.5 ml distilled water and 0.5 ml of 10% $HClO_4$ at 4 °C for 60 min. The homogenates were centrifuged at 10,000 × *g* at 4 °C for 5 min. The precipitated proteins were dissolved in NaOH (1 mM) for protein determination using bovine serum albumin as a standard. To 400 μl supernatant, 100 μl EDTA (10 mM, pH 7.5) and 450 μl of a mixture of freon/trioctylamine (1:1) were added. After centrifugation at 350 × *g* at 4 °C for 2 min 400 μl of the aqueous upper phase were lyophilized. The samples were then dissolved in 0.1 ml of a buffer (pH 6.3) used for cGMP enzyme immunoassay kit; the results were expressed as pmol cGMP formed per mg protein.

4.2.4. Measurement of human platelet aggregation

As described previously [23,24] venous blood was drawn from healthy volunteers who had not taken any drugs known to influence platelet functions within the last 10 days. Blood was anticoagulated with sodium citrate (9 vol. blood + 1 vol. sodium citrate 0.11 M). PRP was prepared by centrifugation at 400 × *g* at room temperature for 12 min. Platelets were counted and adjusted to 2.5–3.0 × 10^8 platelets per ml.

Aggregation was measured turbidimetrically at 37 °C using a four-channel "APACT 4"-aggregometer (Labor, Ahrensburg, Germany). Diazeniumdiolates were added to PRP 2 min before the addition of ADP (5 μM) or collagen (2 μg/ml). Changes in light transmission of the platelet suspension were recorded continuously for maximal 10 min. The extent of aggregation was determined by measuring the maximum amplitude of increase in light transmission. Percentage inhibition of aggregation was calculated from the ratio of extent of aggregation in samples with inhibitors compared to control samples.

4.2.5. Statistics

Data are presented as mean ± S.E.M. from *n* separate experiments. Concentration–response curves were analyzed by non-linear regression using the computer program Origin (Microcal Software, Inc.) and the EC_{50}/IC_{50} values were derived from this analysis. Comparison of means was made using the Student's *t*-test modified according to the Bonferroni method. Differences were considered statistically significant at $P < 0.05$.

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