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Nitric oxide-donating properties of mesoionic 3-aryl substituted oxatriazole-5-imine derivatives

[1]*H. Kankaanranta, †E. Rydell, **Å.-S. Petersson, *P. Holm, *E. Moilanen, ‡T. Corell, ‡G. Karup, *#P. Vuorinen, ‡S.B. Pedersen, **Å. Wennmalm & [2]*T. Metsä-Ketelä

*Medical School, University of Tampere, Finland; †Department of Pharmacology, Faculty of Health Sciences, Linköping University, Sweden; **Department of Clinical Physiology, University of Göteborg, Sweden; ‡GEA Ltd, Copenhagen, Denmark and #Department of Clinical Microbiology, Tampere University Hospital, Finland

1 The nitric oxide (NO)-releasing properties of two new mesoionic 3-aryl substituted oxatriazole-5-imine derivatives (GEA 3162 and GEA 3175) were characterized and compared with the known NO-donors 3-morpholino-sydnonimine (SIN-1) and S-nitroso-*N*-acetylpenicillamine (SNAP).

2 GEA 3162, GEA 3175, SIN-1 and SNAP inhibited adenosine 5'-diphosphate-induced platelet aggregation (IC_{50} values 0.18, 0.39, 3.73 and 2.12 μM, respectively). All four compounds induced a dose-dependent and more than 4 fold increase in cyclic GMP in platelets. The increase in cyclic GMP concentration was potentiated more than 1.5 fold by a phosphodiesterase inhibitor, zaprinast (10 μM) and inhibited 38–97% by oxyhaemoglobin (10–45 μM).

3 All of the four compounds studied converted oxyhaemoglobin to methaemoglobin and formed a paramagnetic NO-haemoglobin complex. All but GEA 3175 formed nitrite and nitrate in phosphate buffer. During a 40 min incubation, GEA 3162, SIN-1 and SNAP (100 μM) produced 50–70 μM $NO_2^- + NO_3^-$ as determined by high performance liquid chromatography. The release of NO and NO_2 by GEA 3175 was increased 140 fold in the presence of human plasma (0.14 and 19.7 ppb in the absence and presence of 1% human plasma, respectively) as analyzed by ozone chemiluminescence.

4 The results suggest that the mesoionic 3-aryl substituted oxatriazole-5-imine derivatives GEA 3162 and GEA 3175 as well as SIN-1 and SNAP release nitric oxide.

Keywords: Mesoionic 3-aryl substituted oxatriazoles; guanosine 3':5'-cyclic monophosphate; nitric oxide; nitric oxide-releasing compounds; platelets

Introduction

Endothelium-derived relaxing factor (EDRF) is a labile substance produced by vascular endothelium (Furchgott & Zawadzki, 1980). Pharmacological and chemical evidence indicates that EDRF is identical with or closely related to nitric oxide (NO), a free radical gas, synthesized from L-arginine by a family of isoenzymes called NO synthases (Ignarro *et al.*, 1987; Palmer *et al.*, 1987; 1988; Knowles & Moncada, 1994). The known biological functions of EDRF-NO include vasodilatation, inhibition of platelet aggregation, neurotransmission and regulation of the immune response. These effects are believed to be mediated mainly by the activation of soluble guanylate cyclase and an increase in the intracellular concentrations of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Moncada *et al.*, 1991; Ånggård, 1994; Wennmalm, 1994; Moilanen & Vapaatalo, 1995).

Organic nitrates are prodrugs which exert their pharmacological actions via a common pathway, i.e. the release of NO. They are metabolized by enzymatic and non-enzymatic processes to release NO in certain tissues, but need thiols as cofactors (Feelisch, 1993). Compounds which spontaneously release NO could overcome some of the problems e.g. development of tolerance related to the classic organic nitrates. Sydnonimines have attracted attention in this respect. 3-Morpholino-sydnonimine (SIN-1) an active metabolite of the anti-anginal drug molsidomine, has been shown to release NO together with superoxide anion ($O_2^{\cdot -}$) (Feelisch, 1991; Hogg *et al.*, 1992). Recently, some chemically different compounds have been reported to release NO directly or indirectly (Maragos *et al.*, 1991; Feelisch *et al.*, 1992; Mülsch *et al.*, 1993; Medana *et al.*, 1994; Kita *et al.*, 1994; Salas *et al.*, 1994).

Mesoionic 3-aryl substituted oxatriazole-5-imine derivatives have recently been synthesized by GEA Ltd. These compounds have been shown to inhibit neutrophil functions (Moilanen *et al.*, 1993; 1994), suppress tumour-cell growth (Vilpo *et al.*, 1994), and to have antibacterial (Virta *et al.*, 1994), vasodilator, antiplatelet and fibrinolytic (Corell *et al.*, 1994) and low density lipoprotein oxidation inhibitory activities (Malo-Ranta *et al.*, 1994). In guinea-pig trachea and human neutrophils the biological activity was associated with an increase in the production of cyclic GMP (Moilanen *et al.*, 1993; Corell *et al.*, 1994). The aim of the present study was to characterize the NO-releasing properties of two 3-aryl substituted oxatriaxole-5-imine derivatives GEA 3162 and GEA 3175. The effects of GEA 3162 and GEA 3175 (Figure 1) were measured on the inhibition of adenosine 5'-diphosphate (ADP)-induced platelet aggregation *in vitro*, production of cyclic GMP in human platelets, conversion of oxyhaemoglobin (oxyHb) to methaemoglobin (metHb) by spectrophotometry, formation of nitrite and nitrate in phosphate-buffer by high performance liquid chromatography (h.p.l.c.) and formation of nitrosyl-haemoglobin (HbNO) complex by electron paramagnetic resonance spectrometry (e.p.r.). The earlier known NO-donors SIN-1 and S-nitroso-*N*-acetylpenicillamine (SNAP) were used as controls. Some of the results of this study were presented as an abstract at the XIII Helsinki University Course in Drug Research (Kankaanranta *et al.*, 1995).

Methods

Platelet aggregation and cyclic GMP production

Platelet rich plasma (PRP) was prepared by centrifugation (150 *g*, 20 min, 20°C) from citrated venous blood from healthy volunteers who had abstained from any drugs for at least 10

[1] Author for correspondence at: Medical School (Bio), University of Tampere, P.O. Box 607, FIN-33101 Tampere, Finland
[2] Deceased September 1995

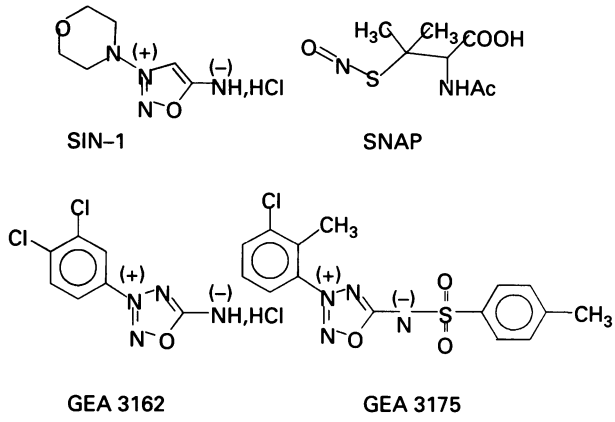

Figure 1 The chemical structures of GEA 3162, GEA 3175, SIN-1 and SNAP.

days. Drugs were added to the PRP ($200-400 \times 10^6$ platelets ml^{-1}) 5 min before platelet aggregation was induced by addition of ADP at threshold concentrations of 2–8 μM. The percentage aggregation during 5 min incubation at +37°C was recorded in a aggregometer setting the 0% response with PRP and the 100% response with the corresponding platelet-poor plasma (Corell *et al.*, 1994).

Cyclic GMP production in platelets was measured as follows: PRP (3.3×10^8 platelets ml^{-1}) was incubated either with or without zaprinast (10 μM) or oxyhaemoglobin (10 μM) for 5 min. Thereafter the compounds studied were added and the incubations continued for 60 s at +37°C. The incubation time was selected on the basis of a time-response curve made with cells from 6 donors (data not shown). The incubations were finished by adding ice cold trichloroacetic acid (final concentration 6%) and samples were centrifuged (10 000 *g* for 10 min). The supernatants were washed four times with water-saturated ethyl ether and stored at −20°C until assayed for cyclic GMP. For cyclic GMP determinations the samples were diluted with an equal volume of 100 mM sodium acetate buffer (pH 6.2) and measured by radioimmunoassay as described earlier (Steiner *et al.*, 1972; Moilanen *et al.*, 1993).

Conversion of oxyhaemoglobin to methaemoglobin

Spectral changes in a solution of the compound tested and 7.1 μM OxyHb in 50 mM Tris-HCl buffer (pH 7.7) at 406 and 411.5 nm wavelengths were recorded at room temperature with a double wave-length spectrophotometer Shimadzu UV-160 A (Shimadzu Corp., Kyoto, Japan). According to the underlying reaction ($HbO_2 + NO \rightarrow MetHb + NO_3^-$), the time-dependent increase in the concentration of MetHb reflects the rate of NO formation. Oxyhaemoglobin was prepared according to Feelisch & Noack (1987), stored in liquid nitrogen and used within 1 h after thawing.

Measurement of nitrite and nitrate by h.p.l.c.

Nitrite and nitrate formed in 10 mM phosphate buffer (pH 7.4) after indicated time at 37°C were measured by h.p.l.c. We used an ODS 2 reverse phase column (Phase Separations Ltd., Deeside, U.K.) with 100 mM NaCl and 10 mM octylamine in H_2O (pH adjusted to 6.2 by H_3PO_4) as a mobile phase (flow rate 1 ml min^{-1}). Nitrite and nitrate were detected by u.v. detector at 225 nm. The detection limit was 0.5 μM for both nitrite and nitrate. $NaNO_2$ and $NaNO_3$ were used as standards.

Measurement of NO and NO_2 by chemiluminescence analyzer

NO and NO_2 formations were measured by chemiluminescence generated in a reaction of ozone with NO (Palmer *et al.*, 1987). The compounds were incubated in 10 mM phosphate buffer (pH 7.4) at ambient temperature. The incubations (50 ml with or without 1% human plasma) were done in a closed vial (100 ml), which were bubbled with air (700 ml min^{-1}). The gas was led through two consecutive cold traps to a Chemiluminescence NO-NO_2-NO_X Analyzer (Model 42, Thermo Environmental Instruments Inc., Franklin, MA, U.S.A.).

Detection of HbNO complex by electron paramagnetic resonance spectrometry

Approximately 1 mg of the compound studied was added to 10 ml of venous blood and kept on ice. Samples of 1 ml were taken successively up to 2 h. Plasma and erythrocytes were separated by centrifugation (12000 r.p.m., 5 min). Erythrocytes were transferred to e.p.r.-tubes and kept in liquid nitrogen until analyzed. The e.p.r. spectra were recorded at 77°K with a Varian e.p.r. spectrometer at a microwave frequency of 9.22 GHz and a microwave power of 10 mW (Wennmalm *et al.*, 1990). Spectra were scanned from 1000 to 3500 gauss with a modulation amplitude of 10 gauss and a scan rate of 125 $Gmin^{-1}$. The reducing agent dithionite was added to see the hyperfine structure (hfs) of the nitrosylhaemoglobin complex at magnetic field between 3000–3500 gauss.

Drugs and chemicals

GEA 3162, GEA 3175, SIN-1 and SNAP were kindly provided by GEA Ltd., Copenhagen, Denmark. They were always freshly prepared in dimethylsulphoxide (DMSO). In experiments the final concentration of DMSO did not exceed 1%. ADP, bovine haemoglobin (Sigma Chemical Company, St. Louis, MO, U.S.A.), dithionite (J.T. Baker, Deventer, The Netherlands), ^{125}I-labelled cyclic GMP (Du Pont NEN Research Products, Boston, Mass., U.S.A.) were obtained as indicated.

Statistics

Results are expressed as mean ± s.e.mean. One-way analysis of variance (supported by Bonferroni significance levels) of the BMDP statistical software (BMDP Statistical Software Inc., Los Angeles, CA, U.S.A.) was used for statistical analysis. Differences were considered significant when $P<0.05$.

Results

Inhibition of platelet aggregation

GEA 3162 and GEA 3175 at submicromolar drug concentrations inhibited ADP-induced aggregation of human platelets (IC_{50} values 0.18 ± 0.02 and 0.39 ± 0.10 μM, respectively). The corresponding IC_{50} values for SIN-1 and SNAP were significantly higher, 3.73 ± 0.43 and 2.12 ± 0.26 μM, respectively ($P<0.001$ when compared to GEA 3162 or GEA 3175). The IC_{50} values of GEA 3162 and GEA 3175 did not differ significantly from each other.

Production of cyclic GMP in human platelets

All four compounds studied increased the cyclic GMP formation in platelets. The increase in cyclic GMP was dose-dependent in the presence of a phosphodiesterase inhibitor, zaprinast (10 μM) (Figure 2). GEA 3162 and SNAP (1 μM) were more potent than equimolar concentrations of GEA 3175 and SIN-1 in increasing cyclic GMP concentration ($P<0.05$ for each comparison). At 10 μM concentrations all drugs were equipotent. In the absence of zaprinast, GEA 3162, GEA 3175 and SNAP (10 μM) increased cyclic GMP more than SIN-1 ($P<0.05$ for each comparison). The increase in cyclic GMP concentration by GEA 3162, GEA 3175, SIN-1 and SNAP (10 μM) was significantly potentiated by zaprinast (10 μM)

(Table 1). OxyHb (10 μM) significantly attenuated the increase in cyclic GMP induced by GEA 3175, SIN-1 and SNAP (Figure 3) whereas that induced by GEA 3162 was attenuated less. However, a higher concentration of OxyHb (45 μM) significantly reduced the increase in cyclic GMP induced by 1 or 10 μM GEA 3162 by 95 and 38%, respectively (Figure 3).

Conversion of OxyHb to MetHb

All the four compounds studied induced a conversion of OxyHb to MetHb. GEA 3162 and SNAP were more potent than GEA 3175 and SIN-1 in oxidizing oxyhaemoglobin in the assay (Figure 4). GEA 3175, although being the least potent, was able to induce a significant ($P<0.05$ for each concentration of GEA 3175) conversion of OxyHb to MetHb as compared to solvent control.

Production of nitrite and nitrate

After incubation of GEA 3162, SIN-1 and SNAP (100 μM) in phosphate-buffer a time-dependent increase in NO_X (nitrite and nitrate) concentrations was detected by h.p.l.c. (Figure 5). Most of the NO_X measured after incubation with GEA 3162, SIN-1 and SNAP was identified as nitrite (generally 60–100% of total NO_X). GEA 3175 did not release measurable amounts of nitrite or nitrate during the 40 min incubation in phosphate-buffer at 37°C. NO_X formation by GEA 3175 was not found in the presence of DMSO (1%), ethanol (up to 10%) or bovine serum albumin (5%).

Production of NO and NO_2

Since we could not find any NO_X formation by GEA 3175 in phosphate-buffer with h.p.l.c., we employed an ozone chemiluminescence method. The release of NO and NO_2 by GEA 3175 (10 μM) was increased 140 fold in the presence of human plasma (0.14±0.82 and 19.69±2.40 ppb in the absence and presence of 1% human plasma, respectively, $n=3$). For comparison GEA 3162 (10 μM) released 6.03±1.84 ppb ($n=4$) in the absence of human plasma.

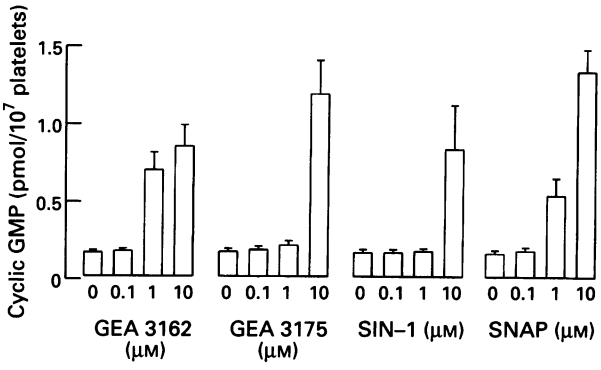

Figure 2 The dose-dependent effects of GEA 3162, GEA 3175, SIN-1 and SNAP on cyclic GMP production in human platelets in the presence of a phosphodiesterase inhibitor, zaprinast (10 μM) during 1 min incubation at 37°C. Results are mean±s.e.mean, $n=6$.

Table 1 The effect of a phosphodiesterase inhibitor, zaprinast on GEA 3162, GEA 3175, SIN-1 and SNAP-stimulated cyclic GMP production in human platelets

	Cyclic GMP (pmol/10^7 platelets)	
	−Zaprinast	*+Zaprinast*
GEA 3162	0.47±0.09	0.91±0.06**
GEA 3175	0.68±0.09	1.14±0.16*
SIN-1	0.18±0.02	1.12±0.19***
SNAP	0.74±0.12	1.56±0.15**

Platelets were incubated for 1 min with the drug (10 μM) with or without zaprinast (10 μM) at 37°C. Results are mean±s.e.mean, $n=6$. The difference between incubations with and without zaprinast is denoted by $*P<0.05$, $**P<0.01$ and $***P<0.001$.

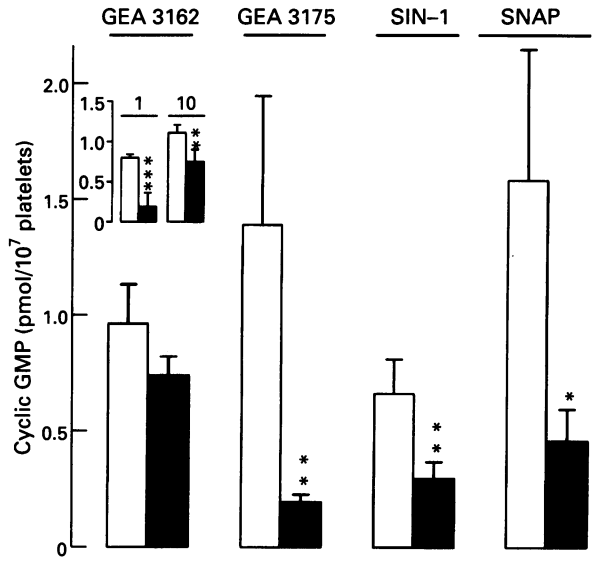

Figure 3 The effect of oxyhaemoglobin (10 μM) on GEA 3162, GEA 3175, SIN-1 and SNAP (10 μM)-stimulated cyclic GMP production in human platelets during 1 min incubation at 37°C. The effect of oxyhaemoglobin (45 μM) on GEA 3162 (1 or 10 μM)-induced cyclic GMP production in platelets is shown in the inset. Results are mean±s.e.mean, $n=6$. The difference between incubations without (open columns) and with (solid columns) oxyhaemoglobin is denoted by $*P<0.05$, $**P<0.01$ and $***P<0.001$.

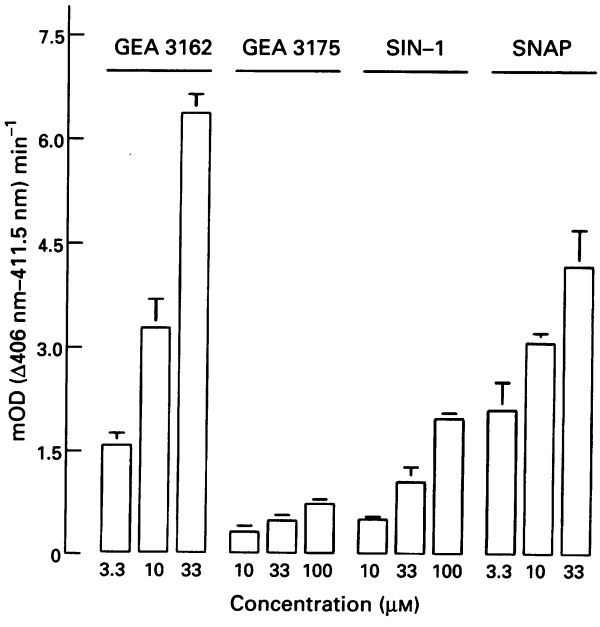

Figure 4 The oxidative effects of GEA 3162, GEA 3175, SIN-1 and SNAP on oxyhaemoglobin. The conversion of oxyhaemoglobin to methaemoglobin was measured with dual-wavelength spectrophotometry at ambient temperature and is expressed as the difference in absorption at 406 and 411.5 nm wavelengths. Results are mean±s.e.mean, $n=3-4$.

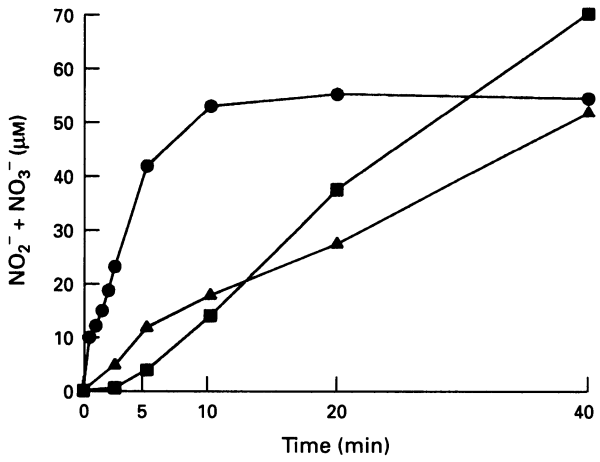

Figure 5 The production of nitrite + nitrate by GEA 3162 (▲), SIN-1 (■) and SNAP (●). Drugs (100 μM) were incubated the indicated time in 10 mM phosphate-buffer at 37°C. Nitrite and nitrate were determined by h.p.l.c. Means of 3–7 determinations are shown. Error bars (generally less than 10%) are omitted for clarity.

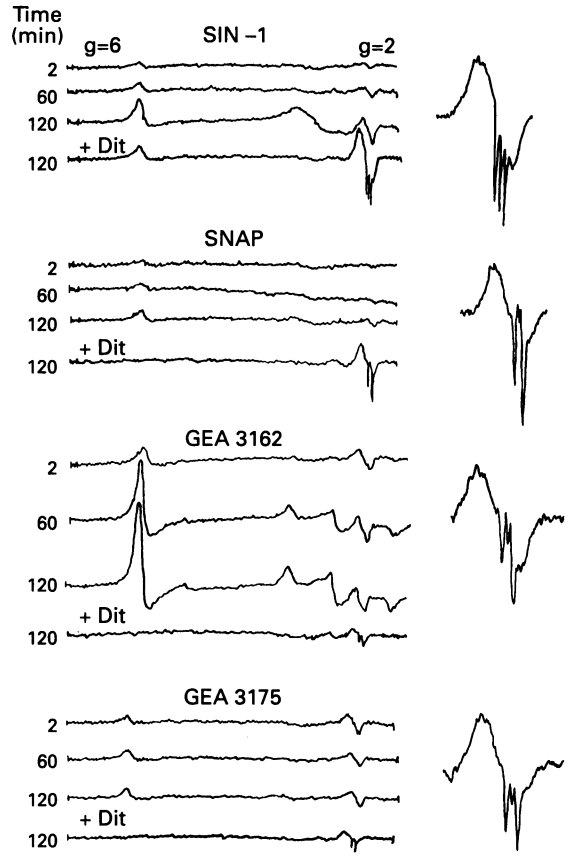

Figure 6 Electron paramagnetic resonance (e.p.r.) signals of nitrosylhaemoglobin (HbNO) of human venous blood treated with GEA 3162, GEA 3175, SIN-1 and SNAP (1 mg 10 ml^{-1} of blood). Typical spectras after 2, 60 and 120 min incubations are shown. Dithionite (Dit) was used to reduce all nitrite to NO and the typical hyperfine structure of HbNO complex was seen (right column).

Formation of HbNO complex

GEA 3162 and GEA 3175 immediately released a compound which gave rise to a nitrosylhaemoglobin-like e.p.r. signal at g = 2.00 (Figure 6). During a 2 h follow-up period the amplitude of this signal was not markedly increased. After addition of dithionite, a hyperfine structure of the complex appeared at g = 2.00, further supporting the identity of the complex as HbNO. The amplitude of the signal was not significantly changed after addition of dithionite. In the case of SIN-1 and SNAP an increase in the HbNO signal amplitude was seen between 2 min and 2 h. Also this signal developed a hyperfine structure typical to HbNO after addition of dithionite. Addition of dithionite to incubates also increased the amplitudes of the HbNO-complexes formed by SIN-1 and SNAP (Figure 6).

Discussion

The present study was designed to prove and characterize the NO-releasing properties of the novel mesoionic 3-aryl substituted oxatriazole-5-imine derivatives GEA 3162 and GEA 3175. Both compounds have been shown to induce an increase in cyclic GMP production in human platelets (Corell *et al.*, 1994) and neutrophils (Moilanen *et al.*, 1993; 1994). For comparison we used two known releasers of NO, namely SIN-1 and SNAP (Feelisch & Noack, 1987; Salas *et al.*, 1994). SIN-1 has been shown to release both NO and superoxide anion which lead to the formation of peroxynitrite (Feelisch, 1991; Hogg *et al.*, 1992).

Platelet aggregation is an event known to be regulated by NO and cyclic GMP (Moncada *et al.*, 1991; Wennmalm, 1994). GEA 3162, GEA 3175, SIN-1 and SNAP inhibited ADP-induced platelet aggregation thus confirming their biological activity. Next, the ability of GEA 3162 and GEA 3175 to increase cyclic GMP in platelets was studied. Both oxatriazole derivatives as well as SIN-1 and SNAP induced a marked increase in cyclic GMP concentration in a dose-dependent manner. This increase was significantly potentiated by a phosphodiesterase inhibitor zaprinast and inhibited by OxyHb. These are biochemical features of NO and suggest that the compounds release NO. Equimolar concentrations of OxyHb were not sufficient to inhibit significantly the rise in cyclic GMP induced by GEA 3162. However, at higher concentrations, OxyHb inhibited the rise in cyclic GMP more pronouncedly. This may be due to the fast decomposition of GEA 3162 in aqueous solutions (Karup *et al.*, 1994).

Binding of NO to oxyhaemoglobin results in oxidation of OxyHb to MetHb in aqueous solutions (Feelisch & Noack, 1987). The MetHb formed can be assayed spectrophotometrically. Both oxatriazole derivatives as well as SIN-1 and SNAP induced the conversion of OxyHb to MetHb. GEA 3162 and SNAP were equipotent in oxidizing OxyHb whereas SIN-1 and GEA 3175 were less effective.

In aqueous solutions NO is readily oxidized to nitrite and nitrate. GEA 3162, SIN-1 and SNAP induced a time-dependent formation of nitrite and nitrate in phosphate-buffer. GEA 3175 did not form measurable amounts of nitrite or nitrate in phosphate-buffer alone or in the presence of DMSO, ethanol or albumin. The lack of formation of nitrite and nitrate in phosphate-buffer as well as the relatively low activity in OxyHb/MetHb assay may indicate a demand for enzymatic degradation of the sulphonamide moiety before NO can be released (Karup *et al.*, 1994). The results may also be complicated with the poor solubility of GEA 3175 in water (less than 1.8 mg l^{-1} in 1% DMSO). Indeed, in those experiments where living cells were present (platelet aggregation, cyclic GMP production and formation of HbNO complex in venous blood) GEA 3175 caused at least as large responses as the other three compounds. To further clarify this issue, we utilized ozone chemiluminescence analyzer for measuring the release of nitrogen oxides. The release of NO and NO_2 by GEA 3175 was increased 140 fold in the presence of diluted human plasma. This may indicate a need for enzymatic degradation or the presence of thiols in order to release NO. This idea is further supported by the recent finding that L-cysteine enhances the release of NO from GEA 3175 (Cohen *et al.*, 1995).

Nitric oxide rapidly reacts with oxyhaemoglobin to form a

HbNO complex. The stability of HbNO complex is far greater than those of HbCO or HbO_2 (Henry *et al.*, 1993). This complex can be detected by electron paramagnetic resonance spectrometry as can the simultaneously formed methaemoglobin (Wennmalm *et al.*, 1990; Archer, 1993). In venous blood both oxatriazole derivatives formed an HbNO-complex detectable with e.p.r. This complex was formed rapidly in 2 min, after which the signal amplitude was not increased during the 2 h follow-up. In the case of SIN-1 and SNAP the amplitude increased during the 2 h incubation. This may indicate a slower release of NO (or a NO-like molecule) by SIN-1 and SNAP than by either of the oxatriazole derivatives. Addition of the reducing agent dithionite (which converts nitrite back to NO) to the incubates changed the amplitudes of the HbNO-complexes formed by SIN-1 or SNAP, but not of those formed by GEA 3162 or GEA 3175. This may indicate that both NO and nitrite were formed in the incubations of SIN-1 and SNAP, while only NO was formed by incubation of GEA 3162 or GEA 3175. The e.p.r. results do, however, not allow any firm conclusions as to whether the different compounds released NO or nitrite. Addition of dithionite also confirmed the existence of a typical hyperfine structure in e.p.r. spectra from all compounds. This demonstrates that the spectra in fact were caused by HbNO.

It has been proposed that the oxyhaemoglobin method is not specific for NO as peroxynitrite has been found to induce a similar spectral change in the assay (Schmidt *et al.*, 1994). SIN-1 is known to release simultaneously NO and $O_2^{\cdot -}$, and to form a peroxynitrite anion (Feelisch, 1991; Hogg *et al.*, 1992). In our assay SIN-1 oxidized OxyHb in a similar manner as reported earlier (Feelisch & Noack, 1987; Schmidt *et al.*, 1994). Thus the possibility exists that the oxatriazole derivatives as well as SIN-1 donate peroxynitrite anion.

In the present study, we found some discrepancy between the ability of GEA 3162, GEA 3175, SIN-1 and SNAP to release NO and to inhibit platelet function *in vitro*. As complications of the methods used one should consider the following facts: the production of both NO and $O_2^{\cdot -}$ by SIN-1, the low solubility of GEA 3175 in water and possible demand for enzymatic degradation or thiols as cofactors in order to release NO. GEA 3175 induced a rise in cyclic GMP concentration in platelets, gave rise to a HbNO complex signal in e.p.r., oxidized OxyHb to MetHb and produced NO and NO_2 as measured by the ozone chemiluminescence. Furthermore, GEA 3175 was reported to release NO in the headspace/NO-analyzer (Karup *et al.*, 1994). Thus the absence of nitrite/nitrate formation in phosphate-buffer cannot be concluded to indicate the absence of NO release from GEA 3175. A chemically related group of vasodilators, furoxans (1,2,5-oxadiazole-2-oxides) has been reported to release NO following chemical reactions with sulphydryl groups of low molecular weight thiols and proteins (Feelisch *et al.*, 1992; Medana *et al.*, 1994). Whether a similar mechanism accounts for the actions of GEA 3175 remains to be seen. We conclude that GEA 3162 releases NO spontaneously in aqueous solutions.

In the present study, we have used several techniques available (Archer, 1993; Kiechle & Malinski, 1993) to detect the presence of NO release from SIN-1, SNAP and the novel mesoionic 3-aryl substituted oxatriazole-5-imine derivatives. In conclusion, we suggest that GEA 3162 and GEA 3175 release NO which is proposed to explain their earlier reported biological activity.

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