ELSEVIER

European Journal of Pharmacology 346 (1998) 97–102

ejp

Radical releasing properties of nitric oxide donors GEA 3162, SIN-1 and *S*-nitroso-*N*-acetylpenicillamine

Päivi Holm [a,b], Hannu Kankaanranta [a,b], Timo Metsä-Ketelä [a], Eeva Moilanen [a,b,*]

[a] *Medical School, University of Tampere, P.O. Box 607 Tampere FIN-33101, Finland*
[b] *Department of Clinical Chemistry, Tampere University Hospital, Tampere, Finland*

Received 29 September 1997; revised 22 December 1997; accepted 30 December 1997

Abstract

The nitric oxide (NO)-, superoxide anion ($O_2^{\cdot-}$)- and peroxynitrite ($ONOO^-$)-releasing properties of 1,2,3,4-oxatriazolium,5-amino-3-(3,4-dichlorophenyl)-chloride (GEA 3162) were characterized and compared with the known NO-donors 3-morpholino-sydnonimine (SIN-1) and *S*-nitroso-*N*-acetylpenicillamine. All the three compounds released NO in aqueous solutions in a dose-dependent manner as measured by ozone-chemiluminescence. GEA 3162 produced more NO than SIN-1, but less than *S*-nitroso-*N*-acetylpenicillamine during a 45 min incubation time. SIN-1 reduced nitro blue tetrazolium and the effect was inhibitable by superoxide dismutase. Reduction of nitro blue tetrazolium was not detected in the solutions of GEA 3162 and *S*-nitroso-*N*-acetylpenicillamine suggesting that SIN-1 but not GEA 3162 and *S*-nitroso-*N*-acetylpenicillamine release $O_2^{\cdot-}$ in their decomposition process. Formation of $ONOO^-$ in solutions of GEA 3162, SIN-1 and *S*-nitroso-*N*-acetylpenicillamine was estimated indirectly by measuring the formation of nitrotyrosine. The data indicate that $ONOO^-$ was produced in the presence of SIN-1 but not in solutions of GEA 3162 and *S*-nitroso-*N*-acetylpenicillamine. The results suggest that GEA 3162 produces negligible amounts of $O_2^{\cdot-}$ and $ONOO^-$ as compared to SIN-1. This adds the value of GEA 3162 as an useful tool in NO research and could well explain the earlier findings on the superior NO-like biological activity of oxatriazole derivatives as compared to SIN-1. © 1998 Elsevier Science B.V.

Keywords: Mesoionic 3-aryl substituted oxatriazole; Nitric oxide (NO); Nitric oxide (NO)-releasing compound; Peroxynitrite; Superoxide; Free radical; Nitrotyrosine; Aminotyrosine

1. Introduction

Nitric oxide (NO) is a free radical gas synthesized by a variety of mammalian cells and tissues including endothelium, smooth muscle, macrophages, platelets and neurons. NO produced by endothelial and neuronal cells regulates blood pressure and mediates non-adrenergic non-cholinergic neurotransmission in a cGMP-mediated process. In activated macrophages NO is involved in the cytotoxic mechanisms reacting with vital enzymes in target cells (Kiechle and Malinski, 1993; Moncada et al., 1991). Organic nitrates exert their pharmacological action via releasing NO after enzymatic conversion or after reactions forming nitrosothiols (Ignarro, 1991; Feelisch, 1993). In addition, groups of chemically different compounds have been reported to release NO directly or indirectly (Kita et al., 1994; Menada et al., 1994; Salas et al., 1994; Kankaanranta et al., 1996).

Superoxide anion ($O_2^{\cdot-}$) is produced by phagocytes as a killing mechanism. Small amounts of extracellular $O_2^{\cdot-}$ may be generated by other cell types and by leakage from normal electron transfer chains (Halliwell, 1995; Crow and Beckman, 1995). NO reacts with $O_2^{\cdot-}$ with a high rate constant to give peroxynitrite ($ONOO^-$) (Beckman et al., 1990) which may spontaneously decompose to yield nitrogen dioxide ($\cdot NO_2$) and hydroxyl radical ($\cdot OH$) (Radi et al., 1993). At physiological pH $ONOO^-$ is a stronger oxidant than $O_2^{\cdot-}$ or NO. It oxidizes lipids, proteins and DNA and nitrates amino acids (Crow and Beckman, 1995; Pryor and Squadrito, 1995). Sydnonimines, like 3-morpho-

Abbreviations: EC, eletrochemical; GEA 3162, 1,2,3,4-Oxatriazolium, 5-amino-3-(3,4-dichlorophenyl)-, chloride; DMSO, dimethyl sulfoxide; HPLC, high performance liquid chromatography; LDL, low-density lipoprotein; NO, nitric oxide; $\cdot NO_2$, nitrogen dioxide; $O_2^{\cdot-}$, superoxide anion; $\cdot OH$, hydroxyl radical; $ONOO^-$, peroxynitrite; SIN-1, 3-morpholino-sydnonimine

* Corresponding author. Tel.: +358-3-215-6741; fax: +358-3-215-6170; e-mail: lleemo@uta.fi

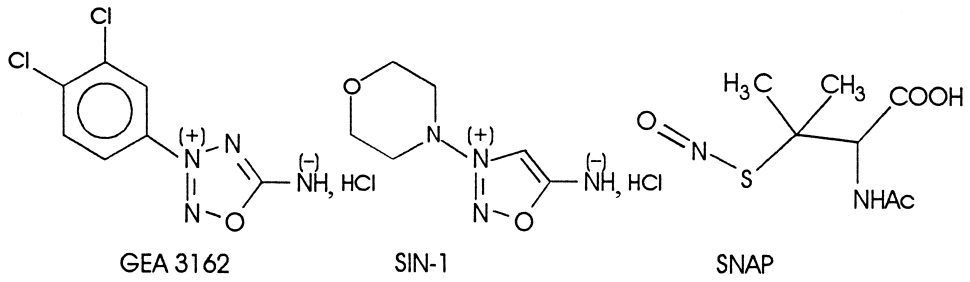

Fig. 1. The chemical structures of GEA 3162, SIN-1 and *S*-nitroso-*N*-acetylpenicillamine (SNAP).

lino-sydnonimine (SIN-1), have been shown to release NO together with $O_2^{\cdot-}$ (Feelisch, 1991; Hogg et al., 1992) leading to the formation of $ONOO^-$. Therefore, SIN-1 may be regarded as a $ONOO^-$ donor rather than a pure NO donor (Hogg et al., 1992).

With the vastly increased research on the biochemistry, pathophysiology and pharmacology of NO, NO-releasing compounds are being employed by many investigators due to the instability and inconvenient handling of aqueous solutions of NO gas. Mesoionic 3-aryl substituted oxatriazole-5-imine derivatives have been shown to release NO spontaneously in buffer solutions (Karup et al., 1994; Kankaanranta et al., 1996) and produce biological activities similar to those of NO (Moilanen et al., 1993; Vilpo et al., 1994; Virta et al., 1994; Corell et al., 1994; Malo-Ranta et al., 1994). Structurally, these oxatriazole derivatives resemble sydnonimines like SIN-1 (Fig. 1) but the chemical reactions leading to the decomposition of these molecules and the release of NO are not known in detail (Karup et al., 1994). In several models, the NO-like biological activity of oxatriazole derivatives is superior to that of SIN-1 (Moilanen et al., 1993; Vilpo et al., 1994, Virta et al., 1994; Corell et al., 1994; Malo-Ranta et al., 1994). This could be due to inactivation of NO in a reaction with $O_2^{\cdot-}$ during the decomposition process of SIN-1. The aim of the present study was to find out whether 1,2,3,4-oxatriazolium,5-amino-3-(3,4-dichlorophenyl)-chloride (GEA 3162), a parent compound of oxatriazole-5-imine derivatives (Karup et al., 1994), releases $O_2^{\cdot-}$ and forms $ONOO^-$ in the NO-producing process. An earlier documented $ONOO^-$ donor SIN-1 and NO-donor *S*-nitroso-*N*-acetylpenicillamine were used as controls.

2. Materials and methods

2.1. Measurement of NO by chemiluminescence analyzer

NO release was measured by ozone-chemiluminescence analyzer (Thermo Environmental Instruments, Model 42). The NO-donors were first dissolved in dimethyl sulfoxide (DMSO) and further diluted in 1:1000 in 50 mM phosphate buffer (pH 7.4) in a glass vial connected to the analyzer via a Tygon-tube.

2.2. Detection of $O_2^{\cdot-}$

$O_2^{\cdot-}$ generation was measured by the superoxide dismutase-inhibitable reduction of nitro blue tetrazolium (Auclair and Voisin, 1985). Drugs were dissolved in DMSO (final concentration 1%) and used immediately in final concentrations of 100 and 500 μM. The reduction of nitro blue tetrazolium was monitored at 560 nm wavelength, at 37°C, for 20 min. Superoxide dismutase (EC 1.15.1.1) at a final concentration of 100 U/ml, was added into the assay cuvette 5 min after the commencement of the incubation, during the linear increase in absorbance.

2.3. Measurement of nitrotyrosine and aminotyrosine by high performance liquid chromatography

$ONOO^-$ nitrates tyrosine into nitrotyrosine which can be measured by high performance liquid chromatography (HPLC) using UV detection at 274 nm as described by Van Der Vliet et al. (1994) and Kaur and Halliwell (1994). To increase sensitivity, nitrotyrosine was reduced to aminotyrosine and measured by electrochemical (EC) detector as described by Darley-Usmar et al. (1992).

Drugs (100 μM) were incubated with 100 μM tyrosine in 10 mM phosphate buffer, pH 7.4, 37°C, for 30 min. The incubates (100 μl) were measured for nitrotyrosine as described by Kaur and Halliwell (1994) or reduced by 10 μl sodium dithionite (1 mM in H_2O, freshly dissolved) and then acidified by 10 μl 5 mM HCl. After centrifugation (3200 g, 2 min), aminotyrosine was analysed by HPLC with an EC-detector (Antec EC-controller, Leyden, the Netherlands). We used an ODS 2 column (5 μm, 25 cm $\times$ 4.6 mm, Phase Separations, Deeside, U.K.) with 1 cm Guard Column (ODS 2, Phase Separations, Deeside, U.K). The eluent (0.8 ml/min) was 50 mM potassium phosphate buffer (pH 3.0) with 20% MeOH (v:v) (Kaur and Halliwell, 1994) and 1.2 g/l heptanesulfonic acid sodium salt monohydrate. 3-Nitro-L-tyrosine was used as a standard in analysis and 3-amino-L-tyrosine was used for the identification of the aminotyrosine peak in HPLC gram.

2.4. Detection of oxygen consumption

Oxygen consumption was measured by a Clark electrode at 37°C (Clark et al., 1953) in 10 mM phosphate buffer. The drugs were first dissolved in DMSO and the low DMSO-induced oxygen consumption was subtracted from counted values.

2.5. Drugs and chemicals

GEA 3162, SIN-1 and *S*-nitroso-*N*-acetylpenicillamine were kindly provided by GEA, Copenhagen, Denmark. Superoxide dismutase, tyrosine, 3-nitro-L-tyrosine and 3-amino-L-tyrosine (Sigma, St. Louis, MO, USA), heptanesulfonic acid sodium salt monohydrate (Fluka Chemie, Buchs, Switzerland), and sodium dithionite (J.T. Baker, Devender, Holland) were purchased as indicated.

2.6. Statistics

Results are expressed as mean ± S.E.M. Statistical significance was calculated by one-way analysis of variance supported by Tukey–Kramer multiple comparisons test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Production of NO

All the three compounds released NO in a time-and dose-dependent manner in phosphate buffer (pH 7.4, 20°C, Table 1). The order of potency at every time point during the 45 min incubation time was *S*-nitroso-*N*-acetylpenicillamine > GEA 3162 > SIN-1. The kinetics of NO production was different between the three compounds. NO release from GEA 3162 and SIN-1 increased continuously during the 45 min follow-up whereas NO release from *S*-nitroso-*N*-acetylpenicillamine peaked at 15 min after dissolved in buffer and decreased thereafter.

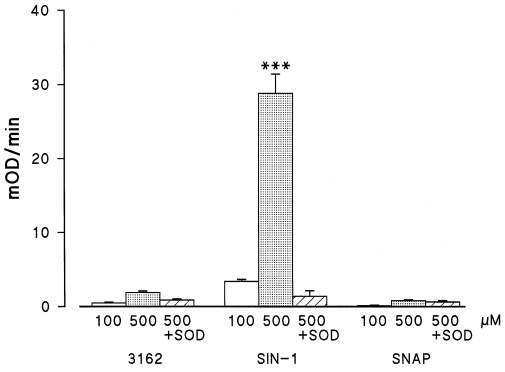

Fig. 2. The ability of NO-donors (100, 500 μM) to reduce nitro blue tetrazoliun in 50 mM phosphate buffer (pH 7.7, 37°C). Mean S.E.M., $n = 4–6$, *** indicates $P < 0.001$.

3.2. Formation of $O_2^{\cdot -}$

The ability of GEA 3162, SIN-1 and *S*-nitroso-*N*-acetylpenicillamine (100–500 μM) to reduce nitro blue tetrazolium is shown in Fig. 2 SIN-1 caused a dose-dependent reduction of nitro blue tetrazolium which was inhibitable by addition of superoxide dismutase. In the presence of GEA 3162 and *S*-nitroso-*N*-acetylpenicillamine there was no significant change in the absorbance indicating negligible, if any, production of $O_2^{\cdot -}$.

3.3. Production of nitrotyrosine and aminotyrosine

To measure the formation of nitrotyrosine as a marker of $ONOO^-$ production, GEA 3162, SIN-1 and *S*-nitroso-*N*-acetylpenicillamine (100 μM) were incubated for 30 min at 37°C in phosphate buffer (pH 7.4) supplemented with tyrosine (100 μM). In the solutions of SIN-1, some nitrotyrosine was found, but the concentrations were near the detection limit of the assay. To increase sensitivity

Table 1
NO release from GEA 3162, SIN-1 and *S*-nitroso-*N*-acetylpenicillamine (SNAP) in 50 mM phosphate buffer measured in the gaseous phase above the buffer by ozone-chemiluminescence analyzer

Drug	μM	NO release (ppb)			
		t (min)			
		2	15	30	45
GEA 3162	10	0.8 ± 0.5	1.7 ± 0.5	1.7 ± 0.4	3.6 ± 1.6
GEA 3162	33	2.0 ± 1.1	7.6 ± 1.4	8.7 ± 1.2	10.1 ± 1.5
GEA 3162	100	9.1 ± 3.1	26.7 ± 6.1	31.8 ± 7.9	43.0 ± 15.5
SIN-1	10	0 ± 0	0 ± 0	0 ± 0	0 ± 0
SIN-1	33	0 ± 0	0.1 ± 0.1	1.4 ± 0.6	3.0 ± 1.0
SIN-1	100	0 ± 0	1.5 ± 0.4	5.0 ± 0.8	7.3 ± 0.8
SNAP	1	11.3 ± 4.7	50.6 ± 16.9	37.8 ± 9.2	30.2 ± 5.5
SNAP	3.3	84.0 ± 23.9	313.9 ± 62.0	228.9 ± 36.3	160.4 ± 19.6
SNAP	10	268.8 ± 63.2	417.4 ± 17.8	379.7 ± 44.3	276.2 ± 83.6

mean ± S.E.M., $n = 5–6$.

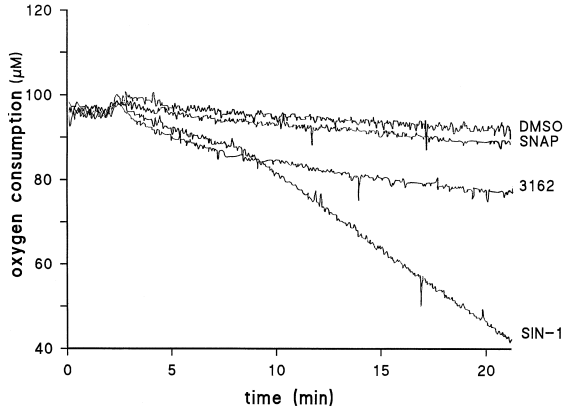

Fig. 3. Oxygen consumption by NO donors (500 μM) in 10 mM phosphate buffer (pH 7.4, 25°C) measured by a Clark electrode. A representative experiment of six.

nitrotyrosine was treated with dithionite to yield aminotyrosine which was detectable with EC detector. Aminotyrosine concentrations in the solutions of SIN-1 were 800 ± 100 nM ($n = 5$). The corresponding value in the solutions of GEA 3162 was about 40 nM which is, however, barely detectable. Aminotyrosine was not found in the solutions of *S*-nitroso-*N*-acetylpenicillamine.

3.4. Oxygen consumption

Fig. 3 shows the oxygen consumption in 500 μM solutions of GEA 3162, SIN-1 and *S*-nitroso-*N*-acetylpenicillamine. The initial rate (during the first minute) of oxygen consumption by SIN-1 (0.7 ± 0.4 μM/min, $n = 6$) was less than that of GEA 3162 (3.2 ± 0.3 μM/min, $n = 6$). Oxygen consumption was not detectable in solutions of *S*-nitroso-*N*-acetylpenicillamine during the first minute. Between 5–20 min after dissolved in buffer, SIN-1 consumed 10 times more oxygen (3.5 ± 0.3 μM/min) than GEA 3162 (0.4 ± 0.1 μM/min) and 20 times more than *S*-nitroso-*N*-acetylpenicillamine (0.2 ± 0.03 μM/min).

4. Discussion

In the present study, the NO-, $O_2^{\cdot-}$- and $ONOO^-$-producing properties of GEA 3162, the parent compound of mesoionic 3-aryl substituted oxatriazole 5-imine derivatives, were characterized and compared with the earlier known NO-releasing compounds SIN-1 and *S*-nitroso-*N*-acetylpenicillamine. SIN-1 releases both NO and $O_2^{\cdot-}$ radicals in aqueous solutions resulting in the formation of peroxynitrite whereas nitrosothiol *S*-nitroso-*N*-acetylpenicillamine releases only NO (Feelisch, 1991; Hogg et al., 1992).

NO released from GEA 3162, SIN-1 and *S*-nitroso-*N*-acetylpenicillamine was measured in the gaseous phase by ozone-chemiluminescence. GEA 3162 released more NO than SIN-1 and less than *S*-nitroso-*N*-acetylpenicillamine. This accords well with our recent study where the NO production by these compounds was detected indirectly by measuring formation of nitrite and nitrate, conversion of OxyHb to MetHb, production of cGMP in platelets and inhibition of platelet aggregation (Kankaanranta et al., 1996). The measure of NO in the gaseous phase probably underestimates the release of NO from SIN-1 as compared to the other NO-donors. The reaction between NO and $O_2^{\cdot-}$ to give $ONOO^-$ is very rapid, the rate constant of this reaction determined by flash photolysis is 6.7×10^9 M^{-1} S^{-1} (Huie and Padmaja, 1993). Thus probably only a minor part of the NO released from SIN-1 reached the analyzer and could be measured in the present experiments.

$O_2^{\cdot-}$ release from the three compounds was measured by nitro blue tetrazolium reduction method (Auclair and Voisin, 1985). SIN-1 was different from GEA 3162 and *S*-nitroso-*N*-acetylpenicillamine in its ability to reduce nitro blue tetrazolium. This activity of SIN-1 was reversed by superoxide dismutase suggesting that it was due to production of $O_2^{\cdot-}$. Our results confirm the earlier reported formation of $O_2^{\cdot-}$ from SIN-1 (Feelisch et al., 1989) and suggest that GEA 3162 and *S*-nitroso-*N*-acetylpenicillamine differ from SIN-1 in this matter.

Reaction between NO and $O_2^{\cdot-}$. under physiological conditions leads to formation of $ONOO^-$ with a fast rate constant (Huie and Padmaja, 1993). $ONOO^-$ is a very reactive molecule and there are no methods to measure $ONOO^-$ as such in physiological conditions. $ONOO^-$ can be assayed indirectly by measuring nitrotyrosine (Kaur and Halliwell, 1994). Using the HPLC method with UV detection described by Kaur and Halliwell (1994) we found some nitrotyrosine in the incubates of SIN-1 but not a trace in those of GEA 3162 or *S*-nitroso-*N*-acetylpenicillamine. To increase sensitivity, we reduced nitrotyrosine to aminotyrosine which could be quantitated by EC detector. The sensitivity was enhanced by factor 60 and there was no doubt of the formation of nitrotyrosine in the incubates of SIN-1. SNAP did not trigger production of measurable concentrations of aminotyrosine. In the incubates of GEA 3162 a barely detectable amounts of aminotyrosine were found. These results confirm the earlier documented formation of $ONOO^-$ in solutions of SIN-1 (Feelisch et al., 1989; Hogg et al., 1992) and suggest that GEA 3162 and SNAP differ from SIN-1 in this characteristic.

Oxygen is consumed in the chemical process leading to NO-release from SIN-1 (Feelisch et al., 1989). Oxygen-dependent and -independent pathways involved in the NO-producing processes of GEA 3162 have been suggested (Karup et al., 1994). In the present study, oxygen consumption in solutions of GEA 3162, SIN-1 and *S*-nitroso-*N*-acetylpenicillamine were measured in conditions where NO was released. The results suggest that SIN-1 and to a lesser extent also GEA 3162 consume oxygen in their

decomposition process. In the case of SIN-1 the oxygen consumption has been associated with the production of $O_2^{\cdot-}$ (Feelisch, 1991). Therefore, the low oxygen consumption in solutions of GEA 3162 is in line with the low $O_2^{\cdot-}$ and peroxynitrite production during NO-releasing process.

Oxatriazole-5-imine derivatives have been shown to have several biological properties typical for NO-donors. Oxatriazole-5-imine derivatives have vasodilator, antiplatelet, fibrinolytic (Corell et al., 1994) and antibacterial (Virta et al., 1994) properties and inhibit oxidation of low density lipoprotein (Malo-Ranta et al., 1994). We have also shown that these compounds inhibit neutrophil functions (Moilanen et al., 1993), leukocyte adhesion to endothelial cells (Moilanen et al., 1994), inhibit lymphocyte proliferation (Kosonen et al., 1997) and suppress tumour cell growth (Vilpo et al., 1994). In most of these studies, the efficacy of the new GEA compounds was compared to the earlier-known NO-donor SIN-1. On molar basis GEA compounds were more potent than SIN-1 in NO-mediated biological actions. This could be explained by the present data suggesting that SIN-1 releases $O_2^{\cdot-}$ in addition to NO whereas $O_2^{\cdot-}$ release from GEA 3162 was negligible. Reaction between NO and $O_2^{\cdot-}$ resulting in the formation of $ONOO^-$ is regarded as an inactivation route for these two reactive radicals (Gryglewski et al., 1986; Pryor and Squadrito, 1995). In addition to this quantitative difference between SIN-1 and GEA 3162 the difference in the $O_2^{\cdot-}$ releasing and $ONOO^-$ producing properties between these two NO-donors is likely to produce qualitative differences in the actions between these NO-donors. For instance, SIN-1 has been reported to initiate lipid peroxidation in low-density lipoprotein (LDL) (Darley-Usmar et al., 1992; Graham et al., 1993) whereas GEA 3162 reduced endothelial cell-mediated oxidation of LDL (Malo-Ranta et al., 1994).

In the present study, we have measured NO release, $O_2^{\cdot-}$ production and $ONOO^-$ formation of mesoionic 3-aryl substituted oxatriazole-5-imine derivative GEA 3162, SIN-1 and *S*-nitroso-*N*-acetylpenicillamine. The results suggest that GEA 3162 releases negligible amounts of $O_2^{\cdot-}$ and $ONOO^-$ as compared to SIN-1 and thus resembles more the NO-donor *S*-nitroso-*N*-acetylpenicillamine. This adds the value of GEA 3162 as an useful tool in NO research and could well explain the earlier findings on the superior NO-like biological activity of oxatriazole derivatives as compared to SIN-1.

Acknowledgements

The study was supported by a grant from The Medical Research Fund of Tampere University Hospital and the Academy of Finland. The skilful technical assistance of Ms. Aija Tuomisto, Mr. Timo Tanhuanpää and Mrs. Heli Määttä is gratefully acknowledged.

References

Auclair, C., Voisin, E., 1985. Nitroblue tetrazolium reduction. In: Greenwald, R.A. (Eds.), CRC handbook of oxygen radical research. CRC Press, Boca Raton, FL, pp. 123–132.

Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A., Freeman, B.A., 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc. Natl. Acad. Sci. USA 87, 1620–1624.

Clark, L.C., Wolf, R., Granger, D., Taylor, Z.J., 1953. Continuous recording of blood oxygen by polarography. Appl. Physiol. 6, 189–193.

Corell, T., Pedersen, S.-B., Lissau, B., Moilanen, E., Metsä-Ketelä, T., Kankaanranta, H., Vuorinen, P., Vapaatalo, H., Rydell, E., Andersson, R., Marcinkiewicz, E., Korbut, R., Gryglewski, R., 1994. Pharmacology of mesoionic oxatriazole derivates in blood, cardiovascular and respiratory systems. Pol. J. Pharmacol. 46, 553–566.

Crow, J.P., Beckman, J.S., 1995. The role of peroxynitrite in nitric oxide-mediated toxicity. Current Top. Microbiol. Immunology 196, 57–73.

Darley-Usmar, V.M., Hogg, N., O'Leary, V.J., Wilson, M.T., Moncada, S., 1992. The simultaneous generation of superoxide and nitric oxide can initiate lipid preoxidation in human low density lipoprotein. Free Radical Res. Commun. 17, 9–20.

Feelisch, M., 1991. The biochemical pathways of nitric oxide formation from nitrovasodilators: appropriate choice of exogenous NO donor and aspects of preparation and handling of aqueous NO solution. J. Cardiovasc. Pharmacol. 17 (Suppl. 3), 25–33.

Feelisch, M., 1993. Biotransformation to nitric oxide of organic nitrates in comparison to other nitrovasodilators. Eur. Heart J. 14 (Suppl. 1), 123–132.

Feelisch, M., Ostrowski, J., Noack, E., 1989. On the mechanism of NO release from sydnominies. J. Cardiovasc. Pharmacol. 14 (Suppl. 11), 13–22.

Graham, A., Hogg, N., Kalyanaraman, B., O'Leary, V., Darley-Usmar, V., Moncada, S., 1993. Peroxynitrite modification of low-density lipoprotein leads to recognition by the macrophage scavenger receptor. FEBS Lett. 330, 181–185.

Gryglewski, R.J., Palmer, R.M.J., Moncada, S., 1986. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. Nature 320, 454–456.

Halliwell, B., 1995. Oxygen radical, nitric oxide and human inflammatory joint disease. Ann. Rheumatic Dis. 54, 505–510.

Hogg, N., Darley-Usmar, V.M., Wilson, M.T., Moncada, S., 1992. Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. Biochem. J. 281, 419–424.

Huie, R.E., Padmaja, S., 1993. The reaction of NO with superoxide. Free Radical Res. Commun. 18, 195–199.

Ignarro, L.J., 1991. Signal transduction mechanisms involving nitric oxide. Biochem. Pharmacol. 41, 485–490.

Kankaanranta, H., Rydell, E., Petersson, A.-S., Holm, P., Moilanen, E., Corell, T., Karup, G., Vuorinen, P., Pedersen, S.-B., Wennmalm, Å., Metsä-Ketelä, T., 1996. Nitric oxide-donating properties of mesoionic 3-aryl substituted oxatriazole-5-imine derivates. Br. J. Pharmacol. 117, 401–406.

Karup, G., Preikschat, H., Wilhelmsen, E.S., Pedersen, S.B., Marcinkiewicz, E., Cieslik, K., Gryglewski, R., 1994. Mesoionic oxatriazole derivates—a new group of NO-donors. Pol. J. Pharmacol. 46, 541–552.

Kaur, H., Halliwell, B., 1994. Evidence for nitric oxide-mediated oxidative damage in chronic inflammation. FEBS Lett. 350, 9–12.

Kiechle, F.L., Malinski, T., 1993. Nitric oxide. Biochemistry, pathophysiology, and detection. Am. J. Clin. Pathol. 100, 567–575.

Kita, Y., Hirasawa, Y., Maeda, K., Nishio, M., Yoshida, K., 1994. Spontaneous nitric oxide release accounts for the potent pharmacological actions of FK409. Eur. J. Pharmacol. 257, 123–130.

Kosonen, O., Kankaanranta, H., Vuorinen, P., Moilanen, E., 1997. Inhibition of human lymphocyte proliferation by nitric oxide-releasing oxatriazole derivates. Eur. J. Pharmacol. 337, 55–61.

Malo-Ranta, U., Ylä-Herttuala, S., Metsä-Ketelä, T., Jaakkola, O., Moilanen, E., Vuorinen, P., Nikkari, T., 1994. Nitric oxide donor GEA 3162 inhibits endothelial cell mediated oxidation of low density lipoprotein. FEBS Lett. 337, 179–183.

Menada, C., Ermondi, G., Fruttero, R., Di Stilo, A., Ferretti, C., Gasco, A., 1994. Furoxans as nitric oxide donors. 4-Phenyl-3-furoxancarbonitrile: thiol-mediated nitric release and biological evaluation. J. Med. Chem. 37, 4412–4416.

Moilanen, E., Vuorinen, P., Kankaanranta, H., Metsä-Ketelä, T., Vapaatalo, H., 1993. Inhibition by nitric oxide-donors of human polymorphonuclear leucocyte functions. Br. J. Pharmacol. 109, 852–858.

Moilanen, E., Arola, O., Malo-Ranta, U., Ylä-Herttuala, S., Vuorinen, P., Mets-Ketel, T., Vapaatalo, H., 1994. Nitric oxide donors inhibit neutrophil adhesion to endothelial cells. In: Moncada, S., Feelisch, M., Busse, R., Higgs, E.A. (Eds.), The Biology of Nitric Oxide, Vol. 4, Portland Press, London, pp. 271–275.

Moncada, S., Palmer, R.M.J., Higgs, E.A., 1991. Nitric oxide: Biochemistry, physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43, 109–142.

Pryor, W.A., Squadrito, G.L., 1995. The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. Am. J. Physiol. 268, 699–722.

Radi, R., Cosgrove, T.P., Beckman, J.S., Freeman, B.A., 1993. Peroxynitrite-induced luminol chemiluminescence. Biochem. J. 290, 51–57.

Salas, E., Moro, M.A., Askew, S., Hodson, H.F., Butler, A.R., Radomski, M.W., Moncada, S., 1994. Comparative pharmacology of analogues of *S*-nitroso-*N*-acetyl-DL-penicillamine on human platelets. Br. J. Pharmacol. 112, 1071–1076.

Van Der Vliet, A., Smith, D., O'Neill, C.A., Kaur, H., Darley-Usmar, V.M., Cross, C.E., Halliwell, B., 1994. Interactions of peroxynitrite with human plasma and its constituents: oxidative damage and antioxidant depletion. Biochem. J. 303, 295–301.

Vilpo, J.A., Vilpo, L.M., Vuorinen, P., Moilanen, E., Metsä-Ketelä, T., 1994. Cytotoxicity of mesoionic oxatriazoles: a novel series of nitric oxide donors. In: Moncada, S., Feelisch, M., Busse, R., Higgs, E.A. (Eds.), The Biology of Nitric Oxide, Vol. 4, Portland Press, London, pp. 286–291.

Virta, M., Karp, M., Vuorinen, P., 1994. Nitric oxide donor-mediated killing of bioluminescent Escherichia coli. Antimicrob. Agents Chemother. 38, 2775–2779.