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Lack of correlation between the observed stability and pharmacological properties of *S*-nitroso derivatives of glutathione and cysteine-related peptides

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Abstract

S-Nitrosothiols (RSNOs) have been widely studied as donors of nitric oxide. In general, RSNOs are considered to be somewhat unstable; however, they are both potent vasodilators and inhibitors of platelet aggregation. In order to improve our understanding of the factors that determine the biological activity of RSNOs, the chemical stability and pharmacological activity of a series of RSNOs was determined. Results show that millimolar solutions of *S*-nitrosocysteine (SNOCys) and *S*-nitroso-L-cysteinylglycine (SNOCysGly) were the least stable, whereas *S*-nitroso-3-mercaptopropionic acid (SNOPROPA) and *S*-nitroso-*N*-acetyl-L-cysteine (SNONAC) were the most stable of the compounds tested. Recent evidence suggests that RSNOs, such as SNONAC, are as unstable as SNOCys at micromolar concentrations. The decomposition of certain RSNOs is catalysed by trace amounts of copper (II) ions, with this phenomenon being particularly evident for SNOCys and SNOCysGly. The decomposition of the more stable RSNOs, including *S*-nitroso-L-glutathione (SNOGSH) and L-γ-glutamyl-L-cysteine (SNOGluCys), were not as sensitive to copper ions. The decomposition of the stable RSNO, SNOGSH, was more rapid in the presence of excess thiol, whereas the decay of the unstable RSNO, SNOCys, was reduced with added thiol. All RSNOs tested inhibited platelet aggregation, relaxed vascular smooth muscle, and inhibited cell growth in the nanomolar range, but their order of potency did not correlate with their chemical stability of millimolar solutions. It is apparent that the potency of an RSNO in a physiological situation will depend on the concentration of the compound present, the presence of trace metal ions such as copper, and the occurrence of transnitrosation reactions.

Keywords: Nitric oxide; *S*-nitrosothiols; Stability; Inhibition of platelet aggregation; Vasorelaxation; Inhibition of cell growth

1. Introduction

RSNOs are an experimental class of NO donors that exert many physiological effects via stimulation of soluble guanylate cyclase, including inhibition of platelet aggregation and vasorelaxation [1,2]. RSNOs have been proposed to be one of the endogenous species that mediate the physiological actions of NO [3–5]. The mechanistic relationship which links the ability of RSNOs to release NO with their pharmacological potency is unclear. Furthermore, the structural features of RSNOs which determine their molecular stability are not well understood. This is illustrated by the difference in stability of a millimolar aqueous solution, between SNOCys, which is exceedingly short-lived, and SNOGSH, which possesses a half-life of hours [6]. In aqueous media, RSNOs can decompose to release NO via several mechanisms. Homolytic cleavage of the *S*-NO bond generates NO and a thiyl radical, although at room temperature and in the absence of appropriate light, this reaction is very slow and decomposition occurs by a copper-catalysed mechanism [7–9]. Alternatively, cleavage can occur by a heterolytic mechanism in the presence of thiolate anions which participate in transnitrosation reactions (i.e. thiol-*S*-nitrosothiol exchange) [10–13]. More recently, Noble *et al.* [14,15] have described a mechanism of RSNO decomposi-

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Abbreviations: NO, nitric oxide; RSNO, *S*-nitrosothiol; CysH, cysteine; SNOGSH, *S*-nitrosoglutathione; SNOCys, *S*-nitrosocysteine; SNOGluCys, *S*-nitroso-L-γ-glutamyl-L-cysteine; SNOCysGly, *S*-nitroso-L-cysteinylglycine; SNOPROPA, *S*-nitroso-3-mercaptopropionic acid; SNONAC, *S*-nitroso-*N*-acetyl-L-cysteine; DTPA, diethylenetriaminpentaacetic acid; and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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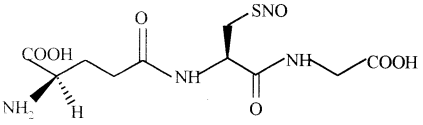
Table 1
Structure of synthetic *S*-Nitrosothiols studied

Structure	*S*-Nitrosothiol	Abbreviation
1	*S*-nitrosoglutathione	SNOGSH
2	*S*-nitrosocysteine	SNOCys
3	*S*-nitroso-L-γ-glutamyl-L-cysteine	SNOGluCys
4	*S*-nitroso-L-cysteinylglycine	SNOCysGly
5	*S*-nitroso-*N*-acetyl-L-cysteine	SNONAC
6	*S*-nitroso-3-mercaptopropionic acid	SNOPROPA



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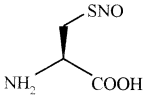

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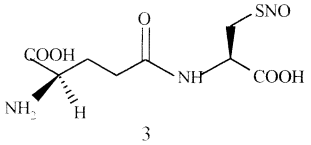

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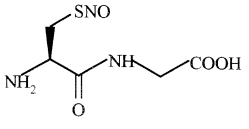

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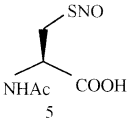

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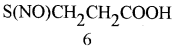

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tion which is dependent on the concentration of disulphide, formed as a result of RSNO breakdown. This group have proposed that certain disulphides will chelate any copper present in solution and in turn increase the half-life of the RSNO being analysed. An additional complicating factor *in vivo* is the potential role which enzymes may play in RSNO decomposition [16–21]. The release of NO from such compounds has been shown to be a combination of some or all of the aforementioned mechanisms [22–24].

The development of RSNOs as potential novel medicines requires a better understanding of factors which determine their stability. To this end, molecular structural features which affect the difference in stability between SNOCys and SNOGSH were investigated. Two *S*-nitroso dipeptides made up of the amino acids which constitute glutathione (GSH), SNOCysGly, and SNOGluCys were synthesised and investigated in terms of chemical stability and pharmacological potency (for structures see Table 1). Their ability to inhibit platelet aggregation, produce vasorelaxation, and inhibit growth of a tumour cell line were compared with those of their congeners SNOGSH and SNOCys. Furthermore, the *S*-nitrosylated forms of 3-mercaptopropionic acid, SNOPROPA, and *N*-acetyl-L-cysteine, SNONAC, were investigated to study further the mechanism of NO release from RSNOs.

Table 2
Molar absorption coefficients and λ_{max} values for the *S*-nitrosothiols studied

S-Nitrosothiol	ε[a] (M^{-1} cm^{-1})	λ_{max}[b] (nm)
SNOGSH	795 ± 15[c]	334
SNOCys	666 ± 57	336
SNOGluCys	918 ± 27	336
SNOCysGly	411 ± 69	337
SNONAC	872 ± 22	335
SNOPROPA	978 ± 95	330

[a] ε values were derived from plots of absorbance versus RSNO concentration, linear regression was carried out on lines, and the ε value was determined from the gradient.
[b] λ_{max} was determined by carrying out a UV scan of a 2.5 mM RSNO standard between 200 and 600 nm.
[c] Mean ± SD for 3 experiments.

2. Materials and methods

2.1. Materials

3-Mercaptopropionic acid was obtained from Aldrich Chemical Co. L-Glutamine, Ham's F12 media, penicillin streptomycin, and Tyrode's salt were purchased from GIBCO, Life Technologies Ltd. Collagen was obtained from Nycomed Arzneimittel GmbH. All other chemicals, including the remaining starting thiols, were purchased from Sigma Chemical Co.

2.2. S-Nitrosothiol synthesis

Solutions of all RSNOs to be used for stability experiments, including SNOCysGly and SNOGluCys, were prepared by the method of Hart [25] by reacting equimolar amounts (500 μM) of thiol, $NaNO_2$, and HCl. *S*-Nitrosothiol synthesis for the pharmacological experiments were carried out in citrate buffer (1 mM, pH 2). The corresponding thiol (20 mM) was dissolved in the citrate buffer and an aliquot (250 μL) was added to citrate buffer (4.5 mL) with an equimolar amount (250 μL, 20 mM) of $NaNO_2$ in saline (0.9% w/v). The concentration of RSNO was assessed by UV spectroscopy taking advantage of the characteristic absorption maxima (λ_{max}) at about 330 nM and using the individual extinction coefficients (ε). ε Values were determined for each RSNO by plotting absorbance at λ_{max} versus RSNO concentration (0.05–2.5 mM). The UV maxima and ε values are listed in Table 2. Due to the light-sensitive degradation of RSNOs, every effort was made in all assays to exclude light.

2.3. Stability studies

Spectrophotometric absorbance changes of the RSNOs in phosphate buffer (1 M, pH 7.4) at 37° were determined at the λ_{max} near 330 nm. In some experiments, the following agents were included in the reaction mixture: DTPA (100 mM), to chelate trace metals, particularly copper. In the case of transnitrosation reactions, GSH or cysteine (CysH) was added to the stability mixture. Where possible, rate constants and half-lives were calculated. The initial rate of the transnitrosation reaction SNOGSH + CysH → SNOCys + GSH was determined at a range of CysH concentrations, and the rate constant (k_1) was obtained from the equation $d[SNOGSH]_0/dt = k_1[SNOGSH]_0[CysH]_0$, where $[SNOGSH]_0$ and $[CysH]_0$ are the initial concentrations of SNOGSH or CysH and $d[SNOGSH]_0/dt$ constitutes the initial rate of SNOGSH decay.

2.4. Platelet aggregation

Platelet aggregation was monitored using the turbidimetric method of Born and Cross [26]. Human blood was collected and prostacyclin-washed platelet suspensions were prepared [27]. Platelet aggregation was induced by collagen (0.1–10 μg/mL) and monitored in a dual-channel aggregometer (Chronolog). All RSNOs were incubated with platelets for 1 min prior to addition of collagen and their effects on platelet aggregation studied for 3 min. Inhibition of aggregation was expressed as the percentage of the maximal aggregation induced by collagen.

2.5. Vasorelaxation studies

Male Wistar rats (250–300 g) were anaesthetized briefly with isofluorane and killed by exsanguination. Rings of the thoracic aorta (3–4 mm), with and without endothelium, were prepared and vasorelaxation studied in the presence of indomethacin (10 μM) as described [28]. Experiments were always carried out on two sets of paired rings. Each set consisted of one ring with the endothelium intact and one denuded of endothelium. Tissues were contracted submaximally (EC_{90}) by addition of phenylephrine (200–900 nm) and cumulative relaxation curves to each RSNO were obtained. EC_{50} responses were expressed as a percentage of the maximum affect of each RSNO from computer-constructed sigmoid logistic dose-response curves.

2.6. Inhibition of cell growth

The ability of RSNOs to inhibit cell growth was assessed in human-derived A549 lung carcinoma cells using the MTT assay [29]. Cells were grown routinely in Ham's F12 medium supplemented with 10% foetal calf serum, penicillin (100 iu mL^{-1}), streptomycin (100 μg mL^{-1}), and glutamine (2 mM). Cells were seeded at 1.1×10^3 cells/well and incubated with RSNOs (0.05–0.5 mM) for 72 hr with exclusion of light, to avoid photochemical degradation. In control incubates, effects of precursors used in the RSNO synthesis were studied.

After the incubation period the cells were washed with PBS (200 μL). MTT was dissolved in medium, (5 mg mL^{-1}), sterilized by filtration, and a volume was added to each well (200 μL). The cells were incubated for a further 4 hr, the medium was removed and the monolayers were then washed with PBS (200 μL). A 200 μL aliquot of buffered DMSO (1 part glycine buffer to 8 parts DMSO) was added to each well and the plates were agitated on a plate shaker for 20 min. Production of formazan, the product of MTT reduction, was measured spectrophotometrically at 540 nm. In each step, the solution was removed from the wells by aspiration to avoid disruption of the cells. Inhibition of cell growth was expressed as the percentage of growth of untreated cells. Despite the short half-lives of some of the compounds studied the cells were incubated with each RSNO for 72 hr to ensure complete formation of the formazan product.

2.7. Data analysis

Relationships between biological stability and pharmacological activity were assessed by comparing the rank order of chemical half-life compared to efficacy in each pharmacological assay. In addition the IC_{50} and EC_{50} values for inhibition of platelet aggregation, inhibition of growth of A549 cells, and vasorelaxation, respectively, were plotted against RSNO half-life. Linear regression analysis was carried out on each line to determine if a correlation existed between the half-life and the pharmacological activity in question.

3. Results

3.1. Chemical stability

SNOCysGly, SNOGluCys, SNOGSH, SNOCys, SNONAC, and SNOPROPA were synthesized and their concentration–time profiles at pH 7.4 were compared. Table 3 shows the decay constants and half-lives for each RSNO under investigation. SNOCysGly, like SNOCys, was short-lived, whilst the stability of SNOGluCys was similar to that of SNOGSH. SNONAC and SNOPROPA were the most stable of the RSNOs investigated. Inclusion into the reaction mixture of DTPA, which chelates copper ions, increased RSNO stability (Table 3). However, the stabilizing effect of copper ion chelation was more pronounced in the case of SNOCys and SNOCysGly than for SNOGSH and SNOGluCys. This fact is reflected by a 24-fold increase in the half-life of SNOCys and an 18-fold increase for SNOCysGly in the presence of DTPA, whereas in the case of SNOGSH and SNOGluCys the half-lives were increased 3- and 10-fold respectively (Table 3). The inclusion of DTPA

Table 3
Chemical decomposition constants (k) and half-lives ($t_{1/2}$) of *S*-Nitrosothiols in the absence or presence of DTPA

RSNO	Without DTPA		With DTPA	
	k (hr^{-1})[a]	$t_{1/2}$ (hr)[a]	k (mmol · dm^{-3} hr^{-1})	$t_{1/2}$ (hr)
SNOGSH	0.0178 ± 0.005	41.6 ± 13.7	0.00306 ± 0.00162	115 ± 62.1
SNOCys	1.35 ± 0.294	0.542 ± 0.151	0.0278 ± 0.003	13.1 ± 1.55
SNOGluCys	0.0165 ± 0.0008	42.2 ± 1.98	0.00274 ± 0.00405	411 ± 329
SNOCysGly	2.22 ± 1.02	0.309 ± 0.103	0.0526 ± 0.004	5.67 ± 0.645
SNONAC	ND[b]	Stable*	ND	Stable
SNOPROPA	ND	Stable	ND	Stable

[a] k and $t_{1/2}$ values for the compounds with measurable half-lives was calculated assuming first-order kinetics, a plot of RSNO concentration versus time for up to 3 half-lives yielded a straight line, the gradient gave k, from which $t_{1/2}$ was calculated, $t_{1/2} = \ln 2/k$. In the presence of DTPA, decomposition seemed to be zero order; in these cases $t_{1/2}$ was calculated from $t_{1/2} = Co/2k$ (Co = initial RSNO concentration).

[b] ND = not determined.

* $t_{1/2} > 500$ hr. Mean ± SD for three experiments.

in solutions of SNONAC and SNOPROPA did not affect their half-lives. Kinetic analysis demonstrated that the rates of decomposition of SNOCys and SNOCysGly were first-order in the absence of DTPA and zero-order in its presence, suggesting that the decomposition of such RSNOs is dependent on a second rate-limiting process, namely the formation of Cu^{+} from Cu^{2+} [9].

In order to elucidate the role of transnitrosation reactions in the decomposition of RSNOs, the decomposition of SNOCys and SNOGSH were studied in the presence of GSH and CysH, respectively. The presence of GSH increased SNOCys stability independently of thiol concentration, stability constants were 3.29 ± 0.716 M^{-1} hr^{-1} in the absence of GSH, and 0.27 ± 0.004, 0.269 ± 0.003, and 0.289 ± 0.003 M^{-1} hr^{-1} in the presence of 0.5, 2.5, and 5 mM GSH, respectively. In contrast, CysH reduced the stability of SNOGSH, in a fashion dependent on CysH concentration. These results are summarised in Fig. 1B, in which the initial rate of SNOGSH decay is plotted versus CysH concentration. It is apparent that low concentrations of CysH very effectively reduced the stability of SNOGSH, but the dependence on [CysH] became markedly lower at greater than equivalent [CysH]. This biphasic phenomenon is probably due to the multiple mechanisms by which thiols can effect the decomposition of RSNOs [34].

3.2. Pharmacological activity

In order to investigate whether the differences in stability described above impact on biological potency, three different parameters of biological activity of the RSNOs were measured: inhibition of human platelet aggregation, vasorelaxation of rat thoracic aorta, and inhibition of growth of the A549 human lung cancer cell line (Tables 4 and 5). All the RSNOs under investigation inhibited platelet aggregation in a concentration-dependent manner. The IC_{50} values observed ranged from 100–800 nM, except for SNONAC, the IC_{50} of which was 3300 nM. Again, all RSNOs tested exhibited concentration-dependent relaxation of vascular smooth muscle. All the compounds, except SNONAC and SNOCys, exhibited an efficacy within the range of 90–800 nM for endothelium-intact rings. As expected, the vasorelaxation potency of RSNOs, except SNOCysGly, was enhanced in rings denuded of endothelium [30]. In the experiments with endothelium-denuded rat aortic rings, all RSNOs exhibited an efficacy within the range of 110 to 620 nM.

With the exception of SNOPROPA, the RSNOs studied were not very potent as inhibitors of growth of A549 cells, with IC_{50} values of 107 ± 34, 144 ± 8, and 181 ± 5 μM for

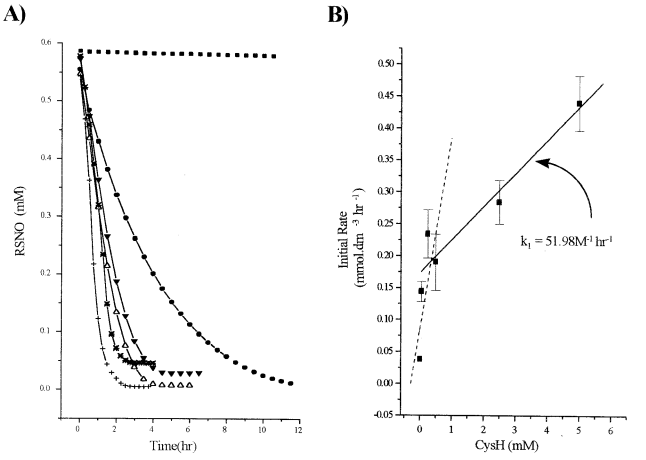

Fig. 1. (A) Representative plot of the stability of SNOGSH (0.5 mM) in the presence of 0 (■), 0.05 (●), 0.25 (△), 0.5 (▼), 2.5 (∗), and (+) 5 mM CysH. Data were obtained as a series of absorbance values for a given time-course. Concentration of RSNO was calculated from absorbance values using the Beer–Lambert Law. The plot is representative of 5 experiments. (B) Relationship between initial rate and CysH concentration for the reaction between SNOGSH and CysH. Rates were measured for each reaction. The initial rate of the transnitrosation between SNOGSH and CysH at a range of thiol concentrations was used to measure the rate constant (k_1) for this reaction determined from $d[SNOGSH]_0/dt = k_1[SNOGSH]_0[CysH]$, where $d[SNOGSH]_0/dt$ is the initial rate of decay of SNOGSH. $[SNOGSH]_0$ and $[CysH]_0$ represent the initial concentrations of SNOGSH or CysH respectively. Each point represents mean ± SD for 3–5 experiments. The two phases observed are represented as (---), phase 1; (—), phase 2.

Table 4
Comparison of half-life, inhibition of human platelet aggregation, and vasorelaxation by *S*-nitrosothiols

RSNO	Half-life (Hr)[a]	Inhibition of Platelet Aggregation[b] IC_{50} (nM)	Vasorelaxation[c]	
			Endothelium-intact EC_{50} (nM)	Endothelium-denuded EC_{50} (nM)
SNOGSH	41.6 ± 13.7	162 ± 103[d]	287 ± 48[e]	148 ± 24[e]
SNOCys	0.542 ± 0.151	172 ± 67	2220 ± 606	617 ± 150
SNOGluCys	42.2 ± 1.98	112 ± 35	825 ± 200	205 ± 41
SNOCysGly	0.309 ± 0.103	794 ± 290	92 ± 30	111 ± 25
SNONAC	>500	3290 ± 918	1365 ± 382	172 ± 38
SNOPROPA	>500	448 ± 225	345 ± 141	138 ± 21

[a] Half-life values in the absence of the copper chelator DTPA, mean ± SD (N = 3).
[b] IC_{50} values were derived from plots of % inhibition versus RSNO concentration. IC_{50} described as the concentration of RSNO required to inhibit aggregation to 50% of the maximum induced by collagen.
[c] EC_{50} values were derived from plots of % relaxation versus RSNO concentration. EC_{50} described as concentration of RSNO required to relax the vessel to 50% of the maximum contraction induced by phenylephrine.
[d] Mean ± SE, N = 5.
[e] Mean ± SE, N = 3–8.

SNOGluCys, SNONAC, and SNOGSH, respectively (Table 5). SNOPROPA appeared to be the most potent agent in the MTT assay, with an IC_{50} value of 43 ± 6 μM. The IC_{50} values for SNOCys and SNOCysGly were >500 μM, with higher concentrations not being studied. In control experiments thiols, from which the RSNOs were derived, did not interfere with cell viability at the concentrations at which the RSNOs inhibited cell growth, excluding the possibility that this effect of RSNOs was mediated by thiol contamination in the RSNO solution or by oxidized thiols formed during RSNO decomposition. These results are summarized in Table 5.

The important aspect of all these pharmacological results is that the effective RSNO concentrations in each assay are within the nanomolar to micromolar range.

3.3. Correlation of stability and pharmacological activity

The rank order of stability of 0.5 mM RSNO solutions at 37°, pH 7.4 and in the absence of DTPA is SNOPROPA = SNONAC ≫ SNOGluCys = SNOGSH ≫ SNOCys > SNOCysGly. In the presence of DTPA, the order of stability is SNOPROPA = SNONAC > SNOGluCys > SNOGSH ≫ SNOCys > SNOCysGly at millimolar concentrations.

Considering the pharmacological responses, the order of potency as inhibitors of platelet aggregation was SNO GluCys > SNOGSH = SNOCys > SNOPROPA > SNO CysGly ≫ SNONAC. These results are shown in Fig. 2A. In the case of vasodilatory action in intact rat aortic rings, the order was SNOCysGly > SNOGSH > SNOPROPA > SNOGluCys > SNONAC > SNOCys (data shown in Fig. 2B). In endothelium-denuded rings, the relative potency differs slightly to SNOCysGly > SNOPROPA > SNOGSH > SNONAC > SNOGluCys > SNOCys. As potential inhibitors of cell growth, the order of potency followed the pattern SNOPROPA ≫ SNOGluCys > SNONAC > SNOGSH > SNOCysGly = SNOCys.

Table 5
Inhibition of growth of A549 cells by the *S*-nitrosothiols studied

RSNO	IC_{50} ± SD (mM)[a]
SNOGSH	0.181 ± 0.005[b]
SNOCys	>0.5
SNOGluCys	0.107 ± 0.034
SNOCysGly	>0.5
SNONAC	0.144 ± 0.008
SNOPROPA	0.0425 ± 0.006

[a] IC_{50} values were derived from plots of % inhibition of cell growth versus RSNO concentration. An IC_{50} dose is described as the dose that gives 50% inhibition of growth of control cells.
[b] Mean ± SD, N = 5.

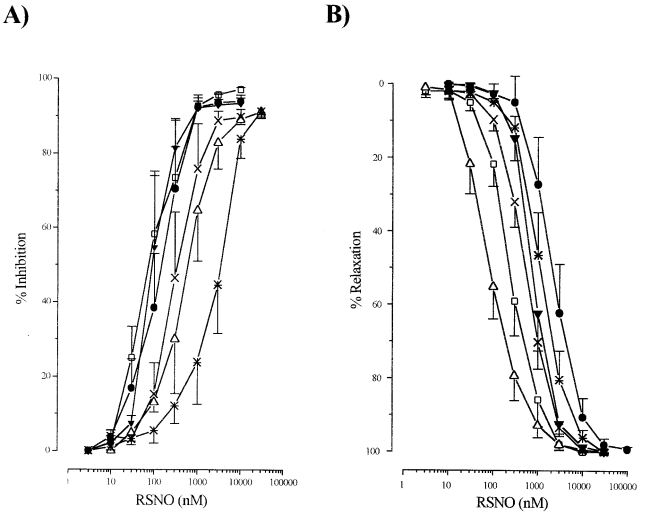

Fig. 2. (A) Inhibition of aggregation of human washed platelets and (B) vasorelaxation of intact rat aortic smooth muscle by SNOGSH (□), SNO-Cys (●), SNOCysGly (△), SNOGluCys (▼), SNONAC (∗), and SNO-PROPA (×). Each point represents the mean ± SE of 3–8 experiments.

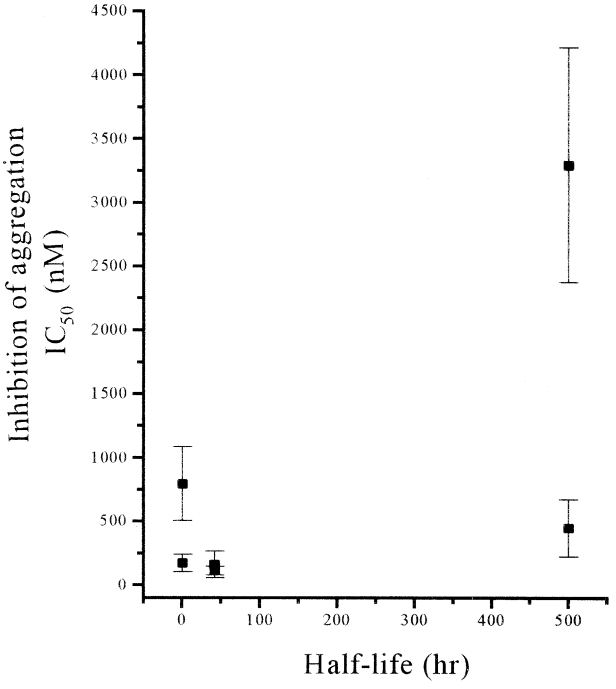

Fig. 3. Correlation between half-life and potency of RSNOs as inhibitors of platelet aggregation.

Linear regression analysis on plots of pharmacological activity versus RSNO half-life clearly showed that no correlation existed between such properties under these experimental conditions. Fig. 3 shows an example plot of RSNO $_{IC50}$ for inhibition of platelet aggregation versus half-life. All results are summarized in Table 6.

4. Discussion

A possible outcome of this study would have been that the less stable RSNOs would be the most potent compounds in inhibiting platelet aggregation and cell growth, and to cause vasorelaxation. The data presented clearly show that the pharmacological activity of RSNOs is not simply related to the stability of the compounds. The possible reasons for the absence of any correlation are discussed below.

Stability studies demonstrated that the chemical stability of SNOGluCys is more than 100-fold higher than that of SNOCysGly, and this difference mimics the stability discrepancy between SNOGSH and SNOCys. Noble *et al.* [14,15] have recently shown that the decomposition of certain RSNOs, in particular SNOGSH and SNOGluCys, are dependent on the starting concentration of RSNO. A mechanism has been proposed, according to which certain disulphides, produced on the decomposition of an RSNO, can complex any Cu^{2+} out of the reaction mixture and hence reduce the formation of Cu^{+} and in turn stabilise the starting RSNO. In the case of SNOGSH and SNOGluCys, at millimolar concentrations these RSNOs are stable as there is sufficient disulphide present to chelate Cu^{2+} out of the reaction mixture. The Cu^{2+} is thought to be chelated by the glutamate residue on the SNOGSH and SNOGluCys. Noble *et al.* [15] have demonstrated that at concentrations between 3×10^{-5}–2×10^{-4} M the stability of SNOGSH is in fact significantly reduced but above this concentration range the RSNO is stable, as was shown in the results in the present study. Considering the remaining RSNOs, the disulphides formed from the decomposition of SNOGSH, SNOGluCys, and SNONAC are able to chelate any free Cu^{2+}, but this is not the case with SNOCys and SNOCysGly. Examining SNOPROPA, it would be expected that this compound would behave like SNOCys, but in fact it was one of the more stable RSNOs investigated. The RSNO concentration-dependency observations would explain how the stable RSNOs such as SNOGSH and SNOGluCys can have such a marked biological efficacy, and in addition would suggest why a correlation was not observed in this study when comparing the stability of millimolar RSNO solutions with pharmacological activities in the nanomolar range. The results obtained with SNOPROPA appear to suggest that the presence of the disulphide is not the only mechanism exerting an influence on the pharmacological activity of RSNOs, regardless of concentration.

SNOCysGly is shown here to share with SNOCys sensitivity towards degradative catalysis by copper ions, whilst SNOGluCys was more resistant to copper-catalyzed degradation, thus emulating SNOGSH. This difference can be rationalized in terms of differential proclivities in the formation of energetically favoured ring structures binding copper, in the form of Cu^{+}, to the nitrogen of the nitroso group and to a second electron-rich atom such as the free amine or oxygen of the carboxylate group as a prelude to efficient degradation [8,9]. Whilst SNOCysGly, like SNOCys, can chelate copper in a six-membered ring con-

Table 6
Relationship between the pharmacological activity and half-life of the *S*-nitrosothiols under investigation

Relationship	r^a	P^a
Inhibition of human platelet aggregation	0.643	0.168
Vasorelaxation in endothelium-intact rat aortic rings	−0.025	0.963
Vasorelaxation in endothelium-denuded rat aortic rings	−0.343	0.506
Inhibition of growth of A549 cells	−0.642	0.17

[a] Linear regression analysis was carried out on plots of the pharmacological activity versus half-life for the RSNOs studied. The analysis yielded a correlation coefficient (r) and a P value for each relationship.

figuration, SNOGluCys, like SNOGSH, can only participate in a larger, energetically less favourable ring structures with Cu^{+}, which precludes the sensitivity towards copper-catalysed degradation.

In addition to the metal ion-sensitive degradation mechanisms described above, other processes may occur depending on the concentration of RSNO present. One of these alternative mechanisms is the cleavage of NO via transnitrosation processes [31]. Thiols seem to be able to increase or decrease RSNO stability, as exemplified by the results described above. The stability of SNOGSH was decreased in the presence of CysH, whilst GSH increased the stability of SNOCys. The reaction between SNOGSH and CysH was first-order with respect to [CysH] at higher concentrations, whereas the reaction between SNOCys and GSH was zero-order with respect to [GSH]. These differential reaction rates in the equilibria between NO donor and recipient may be explained by the relative stability of the starting RSNO. In the case of the SNOGSH/CysH reaction, the relative instability of SNOCys may have driven the equilibrium to the left. With the SNOCys/GSH system, the formation of SNOGSH is again favoured due to the high reactivity of SNOCys, which will drive the equilibrium to the right and hence to the formation of the more stable RSNO. These conclusions are only valid when the starting RSNO is present in millimolar concentrations, since Williams and co-workers have recently shown that micromolar concentrations of RSNOs decompose at very similar rates irrespective of structure [14,15]. With the SNOGSH/CysH system shown in Fig. 1B, there are clearly two phases involved in this reaction, with a rapid decomposition pathway operative at low [CysH]. The details of these biphasic kinetics were not investigated further in this study.

The influence of thiols on the decomposition of RSNOs is complex. It has been shown in certain instances that excess CysH will increase the half-life of various RSNOs, probably by complexation of transition metals [29,30,32]. On the other hand, excess thiol can dramatically increase the rate of NO formation from a number of structurally different RSNOs [33]. This latter effect may be a consequence of the redox function of the thiol rather than the result of a transnitrosation reaction. In conjunction with the previously described theory [8,9] concerning Cu^{2+}-mediated degradation of RSNOs, Dicks *et al.* [34] have observed that when thiols are added to RSNOs in the presence of copper, the thiol may behave as a reducing agent generating Cu^{+} or as a complexing agent for Cu^{2+}, making it less available for reduction.

The results of the pharmacological evaluation of the RSNOs show that differences in stability at millimolar concentrations between RSNOs related to SNOGSH do not translate into differences in pharmacologically relevant concentrations. This conclusion is highlighted by the enormous variation in rank order of potencies of the RSNOs studied, which was established for inhibition of human platelet aggregation, vasorelaxation of rat thoracic aorta rings, before or after removal of the endothelium, and the inhibition of growth of the A549 human lung cancer cell line. The observed discrepancies in rank order were on the whole not predicted by differences in chemical stability. For example, the highly unstable SNOCys was a potent inhibitor of platelet aggregation, but a poor vasorelaxant and did not exhibit any significant inhibition of cell growth at the concentrations studied. The equally unstable SNOCysGly was the most efficacious vasorelaxant of the six agents, but a relatively weak inhibitor of platelet aggregation, and did not show a significant inhibition of growth of A549 cells. The highly stable SNONAC was the least efficacious inhibitor of platelet aggregation, whilst SNOGSH and SNOGluCys, also relatively stable molecules, were powerful inhibitors of platelet aggregation. However, all three agents relaxed rat aortic tone with similar intermediate potency. In contrast, SNOPROPA was one of the least potent RSNOs as an inhibitor of platelet aggregation, a powerful vasorelaxant and the most efficacious inhibitor of cell growth of the RSNOs studied. The only exception to the complete lack of correlation between stability and efficacy is provided by the MTT assay, in which the least stable agents SNOCys and SNOCysGly were both 3–4 times less potent than their less short-lived counterparts, suggesting that the less stable RSNOs decompose rapidly to more innocuous products once in contact with the culture medium, whereas their more stable analogues are able to donate their NO to cellular targets critical in inducing cell death or cytostasis.

The pharmacological activity of RSNOs has been proposed to be mediated by the intact molecule [35], but this hypothesis has been challenged [36]. Alternatively, and more likely, RSNOs release NO *in vivo*, and it is this species which exerts the physiological effects of RSNOs, probably after intermediate generation of endogenous NO carriers via transnitrosation reactions [37–39]. In agreement with this notion, Simon *et al.* [40] have shown that *S*-nitroso proteins such as *S*-nitroso-albumin and *S*-nitroso-tissue type plasminogen activator undergo RSH–RSNO exchange with low molecular weight thiols, i.e. GSH, CysH, and that these protein RSNOs are potent inhibitors of platelet aggregation *in vitro*, *in vivo*, and *ex vivo*.

For the *S*-nitrosothiols described here, the inconsistencies in rank order for chemical stability in the micromolar range on the one hand, and pharmacological potency in nanomolar concentrations on the other, support the notion that RSNO stability is dependent on its concentration. Due to the limitations of methodology used in this study, no firm mechanistic association can be made between stability and pharmacological potency. At concentrations in the micromolar range and above, each RSNO displays a unique profile of susceptibilities towards metal ion-catalysed homolytic cleavage and multiple transnitrosation reactions, causing the liberation of NO. This profile probably has a limited role to play in the physiological activity of these compounds. It is conceivable that at the lower concentrations, more akin to an *in vivo* situation, certain RSNOs such as SNOGSH are unstable enough to release NO more

readily if there is a source of Cu^{2+} readily available. There is likely to be role for transnitrosation or enzymatic breakdown of RSNOs *in vivo*, but these may occur to a lesser extent. In conclusion, the situation *in vivo* is likely to be very complex and hence, it is difficult to make any firm conclusions regarding a correlation between the chemical stability of millimolar concentrations of RSNOs *in vitro* and physiological activity.

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