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In vivo pharmacological activity and biodistribution of S-nitrosophytochelatins after intravenous and intranasal administration in mice

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ABSTRACT

S-nitrosophytochelatins (SNOPCs) are novel analogues of S-nitrosoglutathione (GSNO) with the advantage of carrying varying ratios of S-nitrosothiol (SNO) moieties per molecule. Our aim was to investigate the *in vivo* pharmacological potency and biodistribution of these new GSNO analogues after intravenous (i.v.) and intranasal (i.n.) administration in mice. SNOPCs with either two or six SNO groups and GSNO were synthesized and characterized for purity. Compounds were administered i.v. or i.n. at 1 μmol NO/kg body weight to CD-1 mice. Blood pressure was measured and biodistribution studies of total nitrate and nitrite species (NOx) and phytochelatins were performed after i.v. administration. At equivalent doses of NO, it was observed that SNOPC-6 generated a rapid and significantly greater reduction in blood pressure (~60% reduction compared to saline) whereas GSNO and SNOPC-2 only achieved a 30–35% decrease. The reduction in blood pressure was transient and recovered to baseline levels within ~2 min for all compounds. NOx species were transiently elevated (over 5 min) in the plasma, lung, heart and liver. Interestingly, a size-dependent phytochelatin accumulation was observed in several tissues including the heart, lungs, kidney, brain and liver. Biodistribution profiles of NOx were also obtained after i.n. administration, showing significant lung retention of NOx over 15 min with minor systemic increases observed from 5 to 15 min. In summary, this study has revealed interesting *in vivo* pharmacological properties of SNOPCs, with regard to their dramatic hypotensive effects and differing biodistribution patterns following two different routes of administration.

1. Introduction

Nitric oxide (NO) is an extremely versatile signalling molecule with an extraordinarily diverse array of physiological functions. Disruption to endogenous NO synthesis pathways is a common underlying factor in a variety of pathological conditions, importantly endothelial dysfunction [1,2]. Reduced vascular levels of NO production are responsible for a variety of cardiovascular disorders contributing towards elevated blood pressure, vascular remodelling, and thrombotic events [2]. Exogenous administration of NO *via* NO-donor molecules has been explored as an attractive therapeutic strategy to treat not only cardiovascular disorders [3,4], underpinned by endothelial dysfunction, but also a variety of other pathological conditions, including cancer, infection, osteoporosis, and wound healing [5–8].

The key to successful therapeutic use of NO-donors is achieving targeted NO release and a therapeutically suitable pharmacokinetic profile [9]. Due to the nature of NO as a small, extremely labile and reactive molecule, this objective has proven very challenging to achieve. Interest in the development of appropriate NO delivery systems has increased rapidly across a diverse field of applications [9]. S-nitrosothiols represent a class of NO donors where most have been previously synthesized as S-mono-nitrosothiols based on two different thiol moieties, either penicillamine or cysteine. However, the current trend is the development of di- or poly-S-nitrosothiols in order to increase the payload of compounds releasing NO, thus limiting the drug concentration [10,11]. Poly S-nitrosothiols that have been synthesized include *S,S′*-dinitrosobucillamine ($BUC(NO)_2$), which combines in its structure two *S*-mono-nitrosothiols, *S*-nitroso-*N*-acetylpenicillamine and *S*-nitroso-*N*-acetylcysteine [10]. *S*-nitroso-β-cyclodextrins; a compound that combines photochemically and thermally induced NO release with

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drug carrier ability have been also synthesized where six 6-mono- and 6-multi-*S*-nitroso-β-cyclodextrins (SNO-βCDs) were characterized in terms of their SNO content [12]. Poly-S-nitroso albumin has been developed as a safe and potent multifunctional antitumor agent [13].This study focuses on the *in vivo* activity and pharmacokinetic profiles of a new class of oligopeptide-based NO delivery systems known as S-nitroso phytochelatins (SNOPCs; Fig. 1).

Phytochelatins (PCs) are cysteine-rich oligopeptides produced in plants in response to heavy metals, especially Cd^{2+}, contamination found in soil [14]. Their physiological function is to sequester reactive heavy metals *via* chelation with their cysteine thiol groups, thereby playing a protective role in detoxification. In this way, they are analogous to metallothioneins in mammals. Structurally, PCs are similar to glutathione (GSH), with a primary sequence of $(\gamma\text{-Glu-Cys})_n$ –Gly where usually n = 2–5, but may reach up to 11 in higher varieties of plant species and microorganisms [14,15]. Recent studies have also shown that endogenous NO can react with PCs in plant tissues to produce endogenous mono S-nitrosylated PCs [16]. Interestingly, *in vivo* mono-S-nitrosylation of PCs occurs in a site specific manner, selectively on the single cysteine thiol nearest the N-terminal group. It is thought that this specific S-nitrosylation pattern plays an important role in cell signalling, but to date, little information on the exact nature of such pathways exists [16–18].

SNOPCs may also be utilized as oligopeptide-based NO delivery systems [8,19]. Under *in vitro* conditions, full S-nitrosylation may be achieved, creating an NO delivery system that carries multiple moieties of S-nitrosothiol groups (SNO). Using isolated rat aortic rings, we have previously shown that SNOPCs carrying two-, four or six moieties of SNO (SNOPC-2, -4 or -6) are able to elicit a strong vasodilatory response, equivalent to GSNO at equal molar concentrations of SNO, and more potent than GSNO at equal molar concentrations of compound. However, we observed that SNOPCs are prone to a more rapid physicochemical degradation compared to GSNO and this reduced their biological activity in protracted *in vitro* experiments [19].

As SNOPCs were observed to be excellent transnitrosating agents under *in vitro* conditions [19,20], we were interested in examining their *in vivo* pharmacological activity and biodistribution profiles after intravenous (i.v.) injection. Further, due to a potential therapeutic benefit of inhaled NO donor compounds in diseases such as pulmonary hypertension [21,22], the pharmacokinetics and biodistribution of SNOPCs and GSNO was investigated after intranasal (i.n.) administration. Thus, the aim of this study was to evaluate the pharmacological activity of the SNOPCs as NO delivery systems after i.v. administration and characterize the pharmacokinetic profile of these compounds (both the NO component as well as the oligopeptides carriers) after i.v. and i.n. administration.

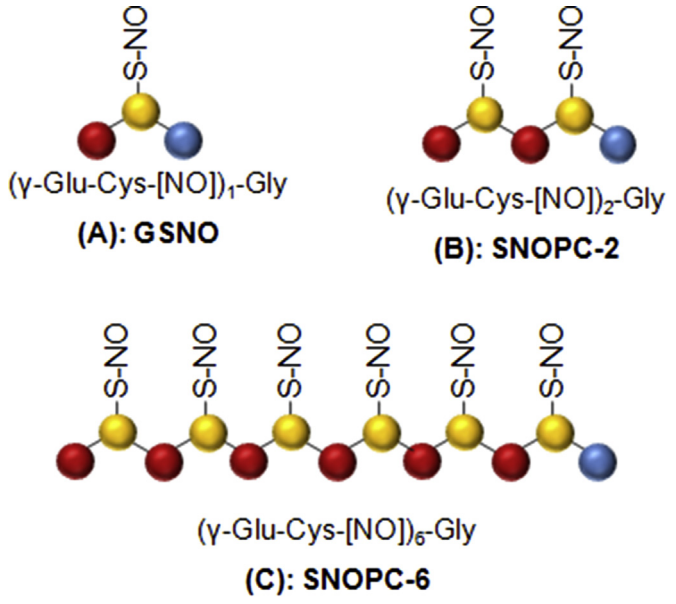

Fig. 1. Schematic structures of (A) GSNO, (B) SNOPC-2 and (C) SNOPC-6.

2. Materials and methods

2.1. Materials

Phytochelatins PC-2 (Mol. wt. = 540.6 Da) and PC-6 (Mol. wt. = 1468.6 Da) were obtained from ANASPEC (Mountain View, CA, USA). Fluorescent-labelled glutathione, PC-2 and PC-6 (conjugated with 7-methoxy coumarin) were obtained from PEPCEUTICALS Limited (Leicestershire, UK). Coomassie Plus (Bradford) Protein Assay reagent was purchased from Thermo Fisher Scientific (Rockford, USA). All other analytical grade chemicals and reagents were purchased from Sigma–Aldrich (Dorset, UK). Mice (male CD-1, 8 weeks old) were purchased from Charles River UK Ltd.

2.2. Synthesis and characterization of GSNO and SNOPCs

Stock solutions of GSNO, SNOPC-2 and -6 were prepared as previously described [20]; an equal volume of acidified nitrite solution ($NaNO_2$/0.9 M HCl) was added to the appropriate glutathione or phytochelatin solution (in 0.9% saline) using equimolar ratios of thiol groups:$NaNO_2$ under conditions where light was excluded. The final pH of the solutions was maintained at 7.4 using 4 mol/L NaOH in 0.1 mol/L Tris buffer. S-nitrosation efficiency (%) was quantified and calculated using UV/Vis absorbance at λ = 334 nm (Cary Varian-300; UK). S-nitrosation efficiencies were 100, 95 and 89% for GSNO, SNOPC-2 and SNOPC-6, respectively. *GSNO and *SNOPCs (*peptides labelled with 7-methoxycoumarin) were S-nitrosated according to the same method and it was determined that the label did not interfere with S-nitrosation efficiency (*GSNO: 98%, *SNOPC-2: 94%, and *SNOPC-6: 88%). All solutions were stored under light exclusion on ice and were administered within 30 min of preparation.

2.3. In vivo studies

All animal experiments were performed under UK Home Office approval according to the Animals Scientific Procedures Act, 1986. Studies were designed and conducted in accord with the ARRIVE guidelines. Experiments were conducted at approximately the same time of day to account for diurnal variation. Drug administration was randomized and unblinded, but data was analyzed by a blinded investigator. Commercially available CD-1 adult male mice (~0.025 Kg body weight) were used for all studies and acclimatized in home cages for 1 week prior to experimentation with *ad libitum* access to food and water and on a 12 h light-dark cycle [23]. Routes of drug administration, surgical protocols and data analysis are detailed below. A total of 180 mice were used, complying with the permitted animal usage outlined in the corresponding Home Office licence. The severity of all procedures was classified as mild.

2.3.1. Haemodynamic measurement of the hypotensive effect of GSNO and SNOPCs in whole animal models

In vivo blood pressure was monitored in anaesthetized animals as previously described [24]. A total of 12 wild type male mice were