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LETTER

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Discovery of *S*-Nitrosoglutathione Reductase Inhibitors: Potential Agents for the Treatment of Asthma and Other Inflammatory Diseases

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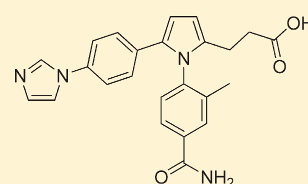
S Supporting Information

ABSTRACT: *S*-Nitrosoglutathione reductase (GSNOR) regulates S-nitrosothiols (SNOs) and nitric oxide (NO) *in vivo* through catabolism of *S*-nitrosoglutathione (GSNO). GSNOR and the anti-inflammatory and smooth muscle relaxant activities of SNOs, GSNO, and NO play significant roles in pulmonary, cardiovascular, and gastrointestinal function. In GSNOR knockout mice, basal airway tone is reduced and the response to challenge with bronchoconstrictors or airway allergens is attenuated. Consequently, GSNOR has emerged as an attractive therapeutic target for several clinically important human diseases. As such, small molecule inhibitors of GSNOR were developed. These GSNOR inhibitors were potent, selective, and efficacious in animal models of inflammatory disease characterized by reduced levels of GSNO and bioavailable NO. **N6022**, a potent and reversible GSNOR inhibitor, reduced bronchoconstriction and pulmonary inflammation in a mouse model of asthma and demonstrated an acceptable safety profile. **N6022** is currently in clinical development as a potential agent for the treatment of acute asthma.



KEYWORDS: GSNOR, GSNO, **N6022**, asthma, pyrrole, nitric oxide

The reaction of glutathione with reactive nitrogen species gives *S*-nitrosoglutathione (GSNO) as a major nitric oxide (NO) metabolite.[1] *S*-Nitrosoglutathione reductase (GSNOR), a member of the alcohol dehydrogenase (ADH) family,[2] has been reported to regulate intracellular *S*-nitrosothiols (SNOs), a biologically important class of stable NO adducts, by reducing GSNO.[3] GSNO has been shown to elicit many biological functions of NO and also serves as a durable depot for NO, which has a short biological half-life.[4] Increases in bioavailable NO are associated with anti-inflammatory and smooth muscle relaxant effects, especially in organ systems characterized by smooth muscle and endothelial/epithelial layers such as the respiratory, cardiovascular, and gastrointestinal systems.[5−7]

GSNOR dysregulation has recently been implicated in respiratory diseases.[8,9] Under normal conditions, NO and GSNO can maintain lung function through their influence on bronchial smooth muscle tone and anti-inflammatory activities.[9,10] In human asthma, there are lowered SNO concentrations in the lungs, likely attributable to up-regulated GSNOR activity.[8] Mice with genetic deletion of GSNOR exhibit increases in lung SNOs and are protected from airway hyperresponsivity.[11] In these mice, GSNOR has been shown to have an important influence on NO containing species, regulation of smooth muscle tone in the airways, and function of adrenergic receptors in lungs and heart.[5,8,12] GSNO also plays an important role in inflammatory bowel disease (IBD). NO and GSNO maintain normal intestinal physiology via anti-inflammatory actions and maintenance of the intestinal epithelial cell barrier. In IBD, reduced levels of GSNO and NO are evident and may also occur via up-regulation of GSNOR activity.[13]

Given such findings, GSNOR has emerged as a potentially important target for the treatment of respiratory, cardiovascular, and gastro-intestinal diseases, many of which have lowered levels of NO as a basis to their pathophysiology.[6,13−15] N30 Pharmaceuticals initiated a discovery effort to identify small molecule inhibitors of GSNOR through high throughput screening of commercially available compounds followed by structure based lead optimization. The goal of the effort was to identify potent GSNOR inhibitors that can be administered either intravenously (IV) or orally (PO) to treat these diseases. In this paper, we report potent GSNOR inhibitors with low nanomolar IC_{50}, which demonstrated *in vivo* efficacy and safety in preclinical models and have advanced into clinical development. We also describe the structure−activity relationships of GSNOR inhibitors and the crystal structure of an enzyme−NAD^+−inhibitor complex, and we report some initial biological and pharmacological properties of these inhibitors.

The majority of GSNOR inhibitors reported in this study were synthesized according to Scheme 1 or 2. Other compounds were

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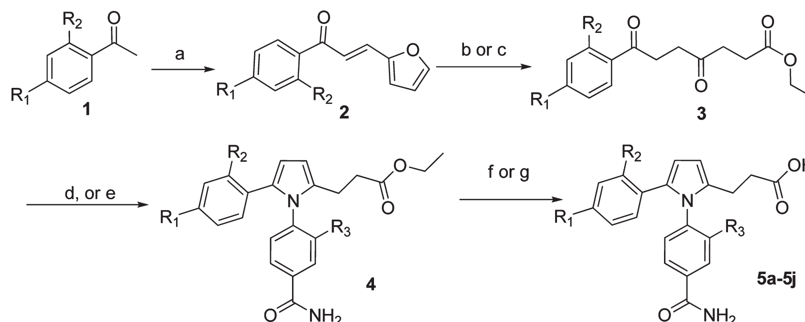
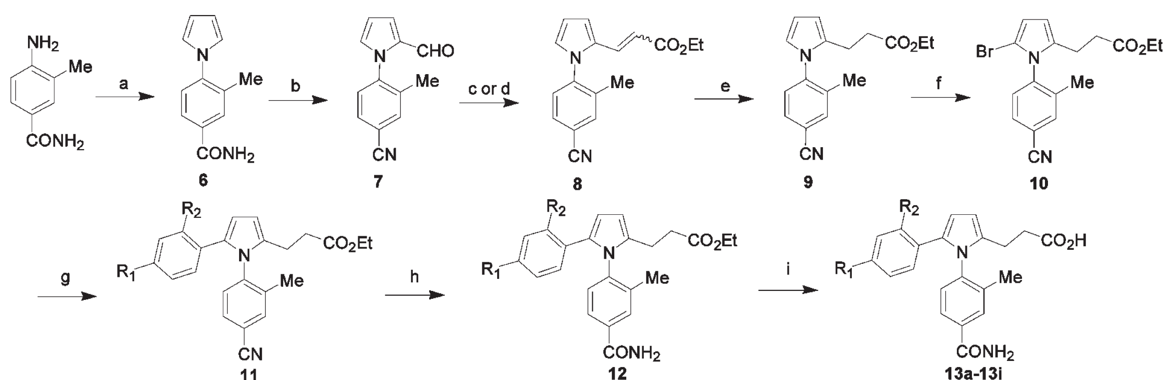
Scheme 1. Synthetic Route of GSNOR Inhibitors[a]



[a] Conditions: (a) 2-furanaldehyde, NaOMe/MeOH. (b) conc HCl/EtOH, reflux. (c) conc HBr/EtOH, reflux. (d) aniline, TsOH/EtOH, reflux. (e) aniline, AcOH/EtOH, reflux. (f) 1 N NaOH/EtOH, 45 °C. (g) 1 N NaOH/MeOH, 45 °C.

Scheme 2. Alternate Synthesis of GSNOR Inhibitors[a]



[a] Conditions: (a) 2,5-dimethoxytetrahydrofuran/AcOH. (b) $POCl_3$/DMF. (c) Ph_3P = $CHCO_2Et$, toluene, reflux. (d) potassium 3-ethoxy-3-oxopropanoate, DMAP/piperidine/AcOH/DMF, 80 °C. (e) H_2, 10% Pd/C in EtOH. (f) NBS. (g) boronic acid, $Pd(PPh_3)_4$, Na_2CO_3. (h) K_2CO_3, H_2O_2/DMSO, room temperature. (i) LiOH.

synthesized using methods described in the Supporting Information.

In Scheme 1, condensation of ketone **1** and 2-furanaldehyde afforded intermediate **2** in high yield.[16] Furan ring-opening of intermediate **2** by HCl or HBr in alcohol under reflux conditions provided diketone **3**.[17] Pyrrole formation was achieved by condensation of diketone **3** and anilines under acidic conditions.[18] Hydrolysis of intermediate **4** by NaOH in alcohol afforded final products **5a**−**5j** in 5−15% overall yield.

Compounds with structural variations on the phenyl ring at the C-5 position of the pyrrole ring were synthesized according to Scheme 2. Condensation of 4-amino-3-methylbenzamide with 2,5-dimethoxytetrahydrofuran in AcOH led to the formation of a key intermediate pyrrole **6**.[19] Formylation of the pyrrole ring of **6** under the Vilsmeier conditions produced **7** in good yield. The acrylate **8** was obtained through a Wittig reaction of **7** with (carbethoxymethylene)triphenylphosphorane[20] or reaction of aldehyde **7** with potassium monoethyl malonate and DMAP in DMF followed by addition of AcOH and piperidine.[21] Hydrogenation of the acrylate **8** using Pd/C afforded the propionic ester **9**, bromination of which resulted in the key building block **10**.[22] Derivatization of **10** with a variety of boronic acids or esters under Suzuki conditions provided intermediate **11**.[23] The nitrile **11** was converted to amide **12** by K_2CO_3 in the presence of H_2O_2 in DMSO. Final hydrolysis under basic conditions afforded **13a**−**13i**, with overall yields of 5−20%.

The GSNOR inhibitors reported here bind reversibly to the HMGSH binding pocket.[24] Although the structures of apo, binary, and ternary complexes of GSNOR have been published,[25] no crystal structure of the GSNO−GSNOR complex has been reported. To guide design and optimization of GSNOR inhibitors, the structures of inhibitors bound to GSNOR and NAD^+ were determined by X-ray crystallography. The crystal structure of the ternary complex of GSNOR, NAD^+, and **N6022** was solved to 1.9 Å (Figure 1) (pdb code: 3QJ5). The enzyme−ligand complex crystallized as a homodimer with N6022 binding to each GSNOR monomer through the following key interactions: (1) the imidazole in the inhibitor interacts with one of the structural zincs, which is also coordinated to histidine 66, cysteine 44, and cysteine 173 to form the zinc coordination tetrahedral; (2) the carboxylic acid of N6022 hydrogen bonds to the glutamine 111 and forms a salt bridge with arginine 114 and lysine 283 from the second monomer; (3) the **N6022** carboxamide hydrogen bonds to glutamine 117; (4) the **N6022** imidazole ring forms a π−π interaction with the nicotinamide ring of NAD^+.

Compound **5a** (GSNOR IC_{50} = 570 nM), which was identified by screening a library of commercially available compounds, was the starting point for lead optimization based on ability to inhibit GSNOR activity *in vitro* (Table 1). Introduction of a methyl group at the R_3-position of the N-phenyl ring as shown in Scheme 1 resulted in slight improvement of IC_{50} from 570 nM to

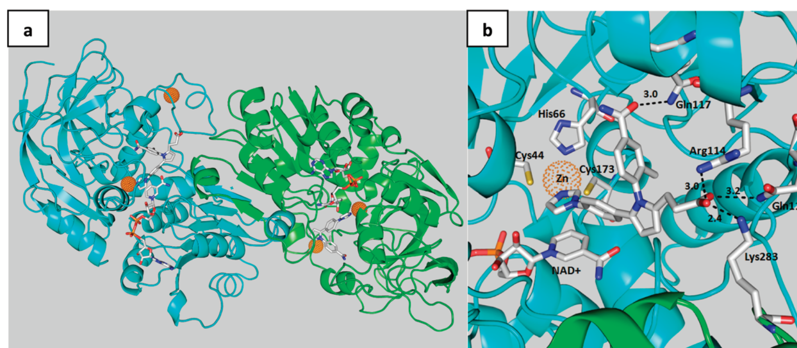


Figure 1. Dimeric structure of **N6022** bound to the GSNOR enzyme−NAD$^+$ complex (a) and the GSNOR binding pocket of **N6022** (b).

Table 1. SAR and PK Properties of GSNOR Inhibitors

compd	R_1	R_2	R_3	GSNOR IC_{50} (nM)	% F	CL (mL/min/kg)
5a	H	H	H	570	86.5	8.9
5b	H	H	Me	360	46.8	11.8
5c	OMe	H	H	460		
5d	OMe	H	F	240		
5e	OMe	H	Cl	190		
14	OMe	H	CF_3	450		
5f	OMe	H	Me	210	16.2	35.4
13a	F	H	Me	500		
13b	Cl	H	Me	120	20.7	16.0
13c	Br	H	Me	160	18.0	21.4
5g	CN	H	Me	563	16.3	25.5
5h	OH	H	Me	160		
13d	CF_3	H	Me	3990		
15	$CONH_2$	H	Me	3920		
N6022	1H-imidazol-1-yl	H	Me	20	4.4	37.8
16	Cl	MeO	Me	56	22.4	20.2
13e	MeO	Cl	Me	200		
17	Br	MeO	Me	96	21.8	22.4
5j	Cl	OH	Me	55	2.72	33.4
13f	Cl	$CONH_2$	Me	160		
18	Cl	EtO	Me	170		
19	Cl	PrO	Me	110		
20	Cl	NMe_2	Me	350		
21	Cl	NHCHO	Me	580		
13g	Cl	Cl	Me	160		
13h	Cl	F	Me	120		
13i	Cl	CF_3	Me	330		

360 nM (**5b**). Substitution at the 4-position of the C5-phenyl ring (R_1) with a methoxy group also improved IC_{50} to 460 nM (**5c**). IC_{50} = 210 nM was achieved by combining both functionalities (**5f**). Structure−activity relationships (SAR) involving substituents at the R_3 position were further explored by replacing the methyl group with halogens (**5d** and **5e**) or a trifluoromethyl group (**14**). Slightly improved activity was observed for the chloro analogue **5e**. The R_1 position was further examined by substitution with a variety of groups. Subtle increases in potency were obtained for the chloro **13b**, bromo **13c**, and hydroxyl **5h** analogues. Significant improvement was realized by substituting the R_1 position with imidazole, leading to **N6022** with IC_{50} of 20 nM. The increased potency of **N6022** can be explained by the fact that the imidazole interacts with one of the structural Zn in GSNOR (Figure 1). Other potential Zn binding moieties including heterocycles were explored, but imidazole appeared to be the best.

SAR involving substituents of the C5-phenyl ring were further explored by substituting at both the ortho and para positions (Table 1). In most cases, the R_1 position was substituted with chlorine for comparison and R_2 was explored for its effect on IC_{50} and pharmacokinetic properties. When R_2 was a methoxy (**16**), IC_{50} = 56 nM was achieved. Interchanging the position of the two groups resulted in a significant loss of potency (IC_{50} = 200 nM, **13e**). The bromo analogue (**17**) of compound **16** also has a low IC_{50} (96 nM). The hydroxyl analogue **5j** has similar activity to its

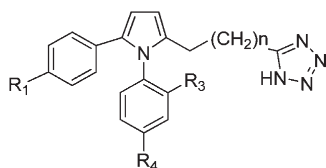


Figure 2. Structures of tetrazole containing GSNOR inhibitors.

Table 2. SAR of Tetrazole Containing GSNOR Inhibitors

compd	R_1	R_3	R_4	*n*	GSNOR IC_{50} (nM)
22	OMe	Me	$CONH_2$	1	1530
23	OMe	Me	$CONH_2$	0	2910
24	Br	Me	$CONH_2$	1	190
25	Br	Me	$CONH_2$	0	560
26	Br	H	OH	1	450
27	Br	H	OH	0	4350
28	1H-imidazol-1-yl	H	OH	1	300
29	1H-imidazol-1-yl	H	OH	0	18

methoxy comparator **16**. Increasing the size of the alkoxy group at the R_2 position resulted in a 2−3-fold reduction in potency (**18** and **19**). Simple nitrogen containing groups did not improve the inhibitory activity (**13f**, **20**, and **21**). Neither disubstitution with halogens (**13g** and **13h**) nor the CF_3 (**13i**) improved inhibitory activity.

Due to the potential metabolic liability of carboxylic acids, this group was replaced with a tetrazole moiety (Figure 2, Table 2), a commonly used bioisostere for carboxylic acid,[26] connecting through the carbon of the tetrazole ring. The data in Table 2 demonstrate that the carboxylic acid can be replaced without a huge loss in potency, particularly when R_1 was bromo (**24**) as compared to its carboxylic acid counterpart **13c** (IC_{50} = 160 nM) or R_1 was the N-imidazole group (**28** and **29**). The length of the chain linking pyrrole and tetrazole (Figure 2) played a critical role. When R_1 was a relatively small group (e.g., methoxy and bromo), better inhibition was achieved with longer alkyl chains (**22**, **24**, and **26**). However, when R_1 was a relatively larger group such as imidazole, the shorter chain length was preferred (**29**), due to size restriction in the binding pocket.

Several compounds were selected for pharmacokinetic studies in mice. The oral bioavailability of the compounds tested ranged from 2.72% to 86.5% (Table 1), and the plasma clearance (CL) after IV administration ranged from 8.9 to 37.8 mL/min/kg. Compound **5a** demonstrated good oral bioavailability (86.5%), although potency was poor. Despite the high potency of **N6022**, its oral bioavailability was very low (4.4%), showing high clearance (37.8 mL/min/kg), potentially due to the high polarity of the imidazole. Compound **16** was the most potent non-imidazole-containing analogue, with an oral bioavailability of 22%.

A battery of *in vitro* assays was employed to assess the potential off-target activity of the GSNOR inhibitors on 54 transmembrane and soluble receptors, ion channels, and monoamine transporters. Off-target effects were estimated from the percent inhibition of receptor radioligand binding at a 10 μM concentration of test compound. Limited off-target activity was observed toward the δ2 opiate receptor for **N6022** (66% inhibition); no other potential off-target activity was detected. Selected compounds were also screened for cytotoxicity toward A549 epithelial lung cells. Minimal cytotoxicity (IC_{50}'s > 100 μM) was observed using this assay. Selected compounds were also screened in cytochrome P450 assays. All compounds screened in these assays exhibited minimal inhibition at 10 μM compound concentration except **N6022**, which had an IC_{50} = 0.77 μM for CYP 2C19. **N6022** was negative in a hERG *in vitro* screen (>100 μM) and showed no evidence of mutagenicity in a bacterial mutagen screen.

Selected GSNOR inhibitors were tested in 5-day toxicology studies in mice. Compounds were administered to mice by IV or PO routes. The selected inhibitors were well-tolerated. The no observable adverse effect levels (NOAELs) for IV administration of **N6022**, **5f**, and **13c** were 30, 50, and 50 mg/kg/day, respectively. The NOAEL for PO administration of **N6022** for 5 days was 100 mg/(kg day).[27]

The efficacy of GSNOR inhibitors was assessed in animal models of asthma and other inflammatory diseases influenced by dysregulated GSNOR. In a mouse model of ovalbumin-induced asthma,[28] **N6022** attenuated methacholine-induced bronchoconstriction (airway hyperresponsiveness) in a dose- and time-dependent manner, with significant efficacy achieved with a single IV dose ≥0.01 mg/kg and when administered from 30 min to 48 h prior to the methacholine challenge. **N6022** also significantly attenuated eosinophil infiltration into the lungs with a single IV dose ≥0.0005 mg/kg.[29] In a mouse model of DSS induced IBD, **N6022** demonstrated significant efficacy with either oral or IV dosing at 1 and 10 mg/kg/day for 10 days. The efficacy of inhibitors **5j**, **5f**, **13c**, and **16** was assessed using one or more of the mouse models of ovalbumin-induced asthma, porcine pancreatic elastase (PPE)-induced COPD, DSS-induced IBD, and a rat model of high dietary salt-induced hypertension (DAHL/S). Significant efficacy was observed across all models.[30−32]

These data suggest that GSNOR inhibitors have potential as therapeutic agents to modulate GSNOR activity and mitigate GSNOR-mediated pathologies *in vivo*. Thus, **N6022** is being advanced in preclinical studies and is currently in phase I clinical trials for the treatment of acute asthma. Other compounds described in this report are under evaluation for treating other types of GSNOR-mediated diseases.

In summary, structure based lead optimization led to a number of potent GSNOR inhibitors with favorable pharmacological properties in multiple disease models. We have identified a potent and reversible GSNOR inhibitor **N6022**, which demonstrated excellent *in vivo* activity in the ovalbumin induced asthma animal model. This compound also demonstrated an acceptable safety profile in the nonclinical toxicology evaluations. **N6022** is currently under further development for its clinical potential to treat acute asthma.

■ ASSOCIATED CONTENT

S Supporting Information. Experimental details and characterization of selected compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

GSNOR, *S*-nitrosoglutathione reductase; GSNO, *S*-nitrosoglutathione; NO, nitric oxide; SNO, nitrosothiol

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