



INHIBITION OF THE ENZYME S-NITROSGLUTATHIONE (GSNO) REDUCTASE DOES NOT CAUSE MECHANISM-BASED TOXICITY

D.B. Colagiovanni, X. Sun, J. Qiu, A. Stout, J. Richards, A. Patton, L. Green and G.J. Rosenthal
N30 Pharmaceuticals Boulder, CO USA

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Contact information:
Dr. Dorothy Colagiovanni
3122 Sterling Circle
Boulder, CO 80301
dot@n30pharma.com

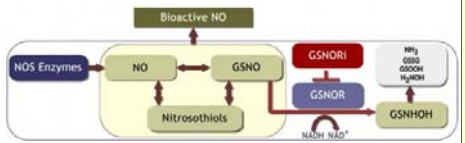
Abstract

S-nitrosoglutathione (GSNO) is an endogenous nitrosol that serves as both a donor and depot for nitric oxide (NO). The enzyme GSNO reductase (GSNOR), also known as alcohol dehydrogenase Class III (ADH III) or formaldehyde dehydrogenase, catalyzes the metabolism of GSNO and controls levels of intracellular S-nitrosolthiols. Given its central role in controlling NO stores and formaldehyde levels, it is important to understand potential negative consequences of modulating GSNO. Fortunately, other enzymes are capable of catalyzing the breakdown of formaldehyde, including ADH 1A1 and 2. Based on studies using exogenous NO donors, NO-mediated effects from reduced breakdown could include hypotension, methemoglobinemia and changes in platelet aggregation. We evaluated potential deleterious effects of GSNO inhibition using small molecule inhibitors, agents being developed for treatment of respiratory diseases. The inhibitory activity of these compounds is in the low nanomolar range. Potency and specificity of the inhibition of GSNO were assessed by *in vitro* enzymatic activity measurements using purified recombinant human GSNO and by comparing compound inhibition of GSNO with other classes of ADH enzymes. *In vitro* screening assays and exploratory rodent toxicology studies were conducted with five representative compounds. Assays included assessments of total GSH, GSH synthesis, lipid peroxidation, mitochondrial function and apoptosis. *In vivo* studies evaluated general toxicity. Results demonstrate that pharmacologic inhibition of GSNO does not adversely impact "on target" endpoints traditionally associated with increased systemic NO and class effects were not seen. *In vitro* assays showed mild toxicity only at exceedingly high doses, while there were no target organs of toxicity in the *in vivo* studies. These findings indicate that inhibiting GSNO does not lead to toxicity and further evaluation of novel inhibitors of the enzyme is warranted.

Introduction

GSNO is one of the primary endogenous sources of bioavailable NO (Figure 1). GSNO and NO concentrations are regulated by GSNO reductase (GSNOR)^{1,2}, and these molecules in turn regulate respiratory function by modulating airway tone and pro- and anti-inflammatory responses in the respiratory tract^{3,4}. Because NO is a labile gas and its endogenous level is difficult to manipulate, it is thought that by preventing the breakdown of GSNO by inhibiting GSNOR, circulating levels of NO and NO-derived species could be regulated and GSNO could have therapeutic value in patients with pulmonary diseases such as asthma or cystic fibrosis. We sought to determine if small molecule inhibitors of GSNOR lead to any mechanism-based toxicity related to excess NO generation.

Figure 1:



In Vitro Materials and Methods

Compounds
Purified human recombinant ADH IB, ADH II and ADH IV were produced in E. coli by deCODE Biosciences (now Emerald Biosciences). The GSNOR inhibitor compounds were synthesized in-house. All other chemicals and reagents were purchased from either Sigma-Aldrich or from VWR.

GSNO Assay
The IC₅₀ values for the inhibitors were determined spectrophotometrically using GSNO as a substrate and by measuring the decrease in absorbance at 340 nm due to the oxidation of NADH to NAD⁺. Final assay conditions were 100 mM NaPO₄, pH 7.4, 0.240 mM GSNO, 0.300 mM NADH, 0.5 µg/ml GSNO enzyme (12.6 nM) and 1% DMSO. The assays were performed using a Shimadzu UV-Visible spectrophotometer and read continuously for 3 minutes. The IC₅₀ values were calculated in GraphPad Prism software using a sigmoidal dose-response (variable slope) curve fitting equation. (TABLE 1).

In Vitro Materials and Methods

ADH IB, ADH II and ADH IV Assays
The IC₅₀ values for the GSNOR inhibitors against ADH IB, ADH II, and ADH IV were determined with ethanol as the substrate. Enzyme activity was determined by the increase in absorbance at 340 nm due to NAD⁺ reduction to NADH. The assays were performed in a 96-well plate and read in a Molecular Devices M2 plate reader. The final assay conditions for ADH IB were: 50 mM NaPO₄ (pH 7.4), 20 µg/ml ADH IB (500 nM), 2 mM NAD⁺ and 2 mM ethanol. The final assay conditions for ADH II were: 90 mM NaPPI (pH 8.9), 4.4 µg/ml ADH II (110 nM), 23.6 mM NAD⁺ and 14.4 mM ethanol. The final reaction conditions for ADH IV were: 25 mM NaPPI (pH 8.8), 2.5 µg/ml ADH IV (63 nM), 7.5 mM NAD⁺, 28 mM ethanol and 1% DMSO. The concentration of ethanol substrate was selected as near the Km as practical so that all modes of inhibition could be detected (TABLE 1).

Off-target Receptor Activity
To evaluate off-target activity of novel compounds, potential secondary pharmacodynamic effects were assessed through a series of receptor binding and enzyme inhibition assays. An evaluation of 80 critical transmembrane and soluble receptors, ion channels, and monoamine transporters was initially made. When off-target activity was identified, IC₅₀ determinations were conducted as follow-up assessments. From the experiments conducted, compounds were shown to interact with the α2 opiate receptor and several kinase enzymes in a concentration range that was ~500-fold greater than its IC₅₀ for GSNO. (TABLE 2)

CeeTox screen
Studies evaluated *in vitro* cytotoxicity in the rat hepatoma cell lines (H4IIE) and assessed measures of membrane integrity, mitochondrial function, cell proliferation, oxidative stress, and apoptosis. The cells were seeded into 96-well plates and cultured in medium containing 20% bovine serum. Following an equilibration period of 48 hr, the cells were treated with the test compound at concentrations of 0, 1, 5, 10, 50, 100, and 300 µM for 24 hr (overnight) at 37°C in 5% CO₂. Camptothecin and rotenone were included as positive control compounds. The cell supernatant or the cells themselves were harvested for biochemical analysis. In addition to monitoring biochemical changes essential for cell health, interaction with P-glycoprotein (Pgp) and solubility were also assessed. The means of each exposure group (n=3-7) were calculated for each assay performed.

hERG Screening
The assay used Opal16, an automated patch-clamp system with a mammalian cell line CHO-K1 that express hERG (human ether-a-go-go-related gene). Data are reported as mean and individual values. The degree of inhibition (%) was obtained by measuring the tail current amplitude before and after drug incubation (the difference in current was normalized to control and multiplied by 100 to obtain the percent of inhibition). Concentration (log) response curves were fitted to a logistic equation (three parameters assuming complete block of the current at very high test concentrations) to generate estimates of the 50% inhibitory concentration (IC₅₀). (TABLE 4).

In Vivo Methods

Exploratory Safety: 5-day studies were conducted with compounds N6446, N6022, N6338 and N6010 (or vehicle hydroxy-propyl-β-cyclodextrin) given QD by IV administration to male CD-1 mice (n=6/gp). Study endpoints included a general health assessment with complete necropsy.

Cardiovascular Assessment: Beagle Dogs (n=1/gp) were dosed with N6022 IV a single time at doses of 5 to 200 mg/kg and blood pressure and cardiac indices were measured.

Results: ADH Activity

TABLE 1 IC₅₀ Values of Novel Compounds with ADH Isozymes

N30 Compound	GSNOR IC ₅₀ (µM)	ADH IB IC ₅₀ (µM)	ADH II IC ₅₀ (µM)	ADH IV IC ₅₀ (µM)
N6001	0.57	>250	ND	>1000
N6022	0.023	21.5	67	0.135
N6338	0.23	>500	68	291
N6446	0.037	>500	ND	>1000
N6501	0.013	>125	ND	0.571

Results: In Vitro Toxicity Screens

TABLE 2 Summary of In Vitro Off-Target Activity of N6022 (<10 µM)

Target	IC ₅₀ (µM)	Fold difference in activity vs. GSNO
α2 opiate (DOP) receptor	4.8	600
p38 α Kinase	4.4	550
CDK2	4.0	500
IKKα/α	4.0	500
ERK2 (P42mapk)	4.2	525
JNK1	3.6	450
FGFR3 kinase	5.3	663
GSK3beta	2.5	313
EphA3 kinase	3.3	413

ND = not determined
CeeTox Screen
Out of these screens, the only noted effect of treatment was a mild decrease in ATP between 10 µM and 300 µM indicative of a change in cellular energy balance. Glutathione (GSH) concentrations were sensitive to treatments with decreases at 300 µM suggesting potential negative effects on intracellular GSH with high test article concentrations (TABLE 3).

TABLE 3 Summary of Changes to GSH and Membrane Integrity

Compound In H411E at 24 hours	Total GSH TC ₅₀ (µM)	Percent Change in Total GSH	Membrane Lipid Peroxidation	Caspase 3 Activity (Index/Dose)
N6010	227	-68.6	0	NC
N6022	288	-52.5	0	NC
N6001	>300	-17.7	0	NC
ROTENONE	0.04	-98.9	0	NC
CAMPTOTHECIN	0.3	-76.2	0	2/30

NC = no change from baseline

TABLE 4 hERG Assay Screen

Compound	Inhibition (IC ₅₀ µM)
N6022	>300
N6446	>100
N6338	>100
N6501	>100
N9949	>100

In Vivo Exploratory Toxicity Study Results

Mouse Study Findings: Animals treated IV with vehicle, N6446, N6022, N6010 or N6338 did not show clinical signs of toxicity over the course of the study (TABLE 5). Body weight changes were not significantly affected by treatments as compared to vehicle treated controls. No gross morphological changes were observed in animals treated with any agent. No compound altered clinical chemistry parameters. The complete blood count measurements showed effects on the erythrogram with N6446. Reductions in red blood cell counts and hematocrit were evident at 50 mg/kg/day IV N6446 administration. No change in methemoglobin levels or platelet function was evident with any compound.

Organ weights were unaffected with compounds N6010 and N6338. Compounds N6446 caused increased liver weights with 50 mg/kg/day administration. The weight increase corresponded with an increase in splenic and hepatic extramedullary haematopoiesis, mild hepatocellular degeneration and kupffer cell hypertrophy. N6022 caused an increase in adrenal weights at 60 mg/kg/day with no corresponding histopathology.

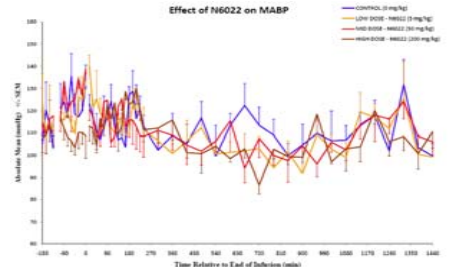
In Vivo Exploratory Toxicity Study Results

TABLE 5. Summary of In Vivo Findings

Agent (IV Dose/Day)	Clinical Obs	Body Weight Effects	Organ Weight Effects	Clinical Pathology Findings	Microscopic Evaluation
N6446 50 mg/kg	-	-	↑Liver wt ↓Spleen wt	↓JRC count ↓Hemoglobin ↑Hematocrit ↓Platelets	↑EMH in liver Kupffer cell hypertrophy Hepatocellular periportal atrophy
N6022 60 mg/kg	-	-	↑Adrenal wt	-	-
N6010 60 mg/kg	-	-	-	-	-
N6338 50 mg/kg	-	-	-	-	-

Dog Cardiovascular Findings: N6022 was assessed for effects on cardiovascular function. No effects were evident with treatment up to 200 mg/kg on heart rate or blood pressure indices (representative data shown in Figure 2).

Figure 2 Blood Pressure Measurements in Beagle Dogs



Conclusions

- With GSNOR inhibitors there was no notable activity against either ADH IB or ADH II isozymes; however, there was observed activity against ADH IV, but of a magnitude significantly less than for ADH III.
- There was only modest off-target activity. Most of the observed off-target activity were related to kinases including p38 alpha and delta, JNK1, and IKKα. Inhibitory effects of N6022 were observed at concentrations exceeding 2 mM, ~500-fold less selective than for GSNOR.
- A change in total GSH was noted with several of the compounds, but at concentrations exceeding 200 µM, not physiologically relevant.
- The liver was a target organ for several compounds, with substantial therapeutic indices relative to efficacy.
- The cardiovascular assessments did not yield any adverse findings related to excess NO generation.
- No significant adverse effects related to mechanism-based toxicity were found.

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