Author's response to reviews

Title: Pharmacologic inhibition of S-nitrosoglutathione reductase protects against experimental asthma via both bronchodilatory and anti-inflammatory activities

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Author's response to reviews: see over
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Dr. Timothy Shipley
Executive Editor, BMC Pulmonary Medicine

Re: Manuscript # 4470665078391576; Response to Peer Review

Dear Editor,

Please find attached our manuscript, Blonder et al., “Pharmacologic inhibition of S-nitrosogluthathione reductase protects against experimental asthma in BALB/c mice via both bronchodilatory and anti-inflammatory activities” which has been revised to address questions and comments from the editor and the three peer reviewers. In addition, we have provided a point-by-point response to the concerns below.

We trust that we have adequately addressed these concerns in our responses as well as within the revised manuscript. We therefore appreciate your further review of our manuscript and look forward to your decision in regard to publication in your journal.

Sincerely,

Joan P. Blonder

Point-by-Point Response to editor and peer reviewers:

Editorial Requirements:

1. Could you please specify the names of the ethics committees that approved the animal study and mention this in the manuscript.

   The mouse OVA studies were conducted at Bio-Quant/Apricus Biosciences in San Diego, CA. This CRO now operates as BioTox Sciences. The ethics committee that approved the mouse OVA studies was Bio-Quant’s Institutional Animal Care and Use Committee (IACUC) and attending veterinarian following guidelines provided under the United
States Department of Agriculture (USDA) Animal Welfare Act (AWA) and with approval of the Office of Laboratory Animal Welfare (OLAW). We have edited the Animal portion of the Methods section to include this more detailed information and for clarity.

Reviewer 1:

1. Major Compulsory Revisions that The Authors must clarify.
   a. Which are the functional characteristics of N6022. What's about possible adverse effect?

   Additional characteristics regarding the function and potential adverse effects for N6022 have been reported in other manuscripts from our group. N6022 is a fully reversible inhibitor and binds within the enzyme:substrate binding pocket. N6022 potently and selectively inhibits GSNOR with an IC\textsubscript{50} of 20 nM and a Ki of 2.3 nM (Sun et al 2011, ACS Med Chem Lett, 2:402; Green et al 2012, Biochem, 51:2157).

   A 14 day toxicology study in rats demonstrated that IV N6022 was well-tolerated at doses up to 50 mg/kg/day and that no biologically significant adverse findings were noted up to a dose of 10 mg/kg/day (Colagiovanni et al 2012, Regul Toxicol Pharm, 62:115).

   Of note, N6022 has been tested by the IV route in two Phase 1 studies in healthy subjects, in an exploratory Phase 2 study in mild asthmatics, and is currently being tested in a Phase 1 study in cystic fibrosis patients homozygous for the F508del-CFTR mutation. A thorough battery of safety pharmacology and GLP toxicology studies were conducted to support the clinical testing of IV N6022. No severe adverse effects have been noted with the testing of N6022 in humans to date.

   We have included more detail in regard to the functional characteristics and safety of N6022, as well as the above references within the Methods of the manuscript.

   b. How many mice in every experimental models?

   The number of mice per group had been included in the legends to each figure. For clarification, we included more detail within the legend to Figure 1.
c. The rationale for averaging Penh in 4 minutes between two different Mch nebulization, when every Mch nebulization last 5 minutes and the recovery time is only of one?

The protocol for Penh calculation used is based on the nature of the data obtained by the CRO performing the assay. The Penh calculation takes into account a sufficient lag time upon introduction of each MCh challenge dose, allows for calculation of the Penh response across a majority of the time frame at each exposure level, and allows for calculation using data that are within the ‘middle’ of the data set.

d. Why N6022 activity works in different manner between OVA.sensitized or Not mice?

N6022 likely works in a different manner between the OVA-sensitized and non-sensitized mice due to the potent anti-inflammatory actions exerted by N6022. We had included text in regard to this reasoning within the Discussion.

More specifically, N6022 may exert different biological influences in disease vs. non-disease states through restoration of GSNO and the SNO pool, a beneficial activity in disease states, but likely of no consequence or a less effective mechanism in non-disease states – that is, the levels of GSNO and GSNO/SNO mediated functions are normal and sufficient and not further altered by inhibition of GSNOR with N6022.

In support of this hypothesis, we have recently demonstrated that treatment of rats with a related GSNOR inhibitor decreases blood pressure and nitric-oxide dependent flow mediated vasodilation in a salt-induced hypertensive rat model, whereas no effect of the GSNOR inhibitor is noted in normotensive rats (Chen et al, 2013).

We have added more detail and this reference within the discussion to clarify these points.

e. The discussion and conclusions are well balanced and adequately supported by the data. The limitations of thw work must be better clearly stated.

We have edited the discussion and included more detail in regard to the limitations of the work for clarity and as requested by Reviewer 3 in Points #1 and #2.

The title and abstract must contqain the word "in balb-mice".

The title has been edited to include the species and strain.
Reviewer 2:
Discretionary Revisions:

1. High levels of myeloperoxidase and MMP-9 suggest that neutrophils may be elevated at the time of therapeutic intervention in this model. Differential cell counts were performed, but only eosinophil counts were reported. It would be more informative if total cell counts and differentials (e.g. eosinophils, macrophages, neutrophils) were reported. This would allow correlation between BAL cells and inflammatory cytokines/chemokines.

   We appreciate your suggestion and agree that it would be meaningful to correlate BALF biomarkers and cell types. However, differential cell counts were not performed in these studies. We were initially interested in the eosinophilic response as these are the main inflammatory cell type in this model. We did perform differential cell counts in later studies in the OVA model while testing other GSNOR inhibitors and showed inhibition of BALF neutrophils. However, these analyses pertain to other GSNOR inhibitors and thus are not relevant for including within this manuscript.

2. The inclusion of bioassay data from naïve rat trachea (Fig. 5) seems a little out of place, since the rest of study is focused on mouse allergic asthma model. Authors should consider removing this data.

   Although the rest of the study does focus on the mouse allergic asthma model, we opt to retain Figure 5 data within this manuscript. We feel that these data from the naïve rat trachea provide additional support in regard to the ability of N6022 to exert effect on airway smooth muscle in a relevant, commonly employed model. We also have included a panel of data showing the ability of N6022 to relax airway smooth muscle following ‘pre-contraction’ with methacholine in this model. These additional data were included to further support the ability of N6022 to exert bronchodilatory effect in response to Reviewer 3 Point #5.

Minor Essential Revisions:

1. Background, p.5 (paragraph 1), it is unclear what “durable” means when in reference to GSNO and SNOs. A clearer description is needed.

   We have deleted the word ‘durable’ from this sentence which now reads as ‘GSNO and SNOs serve as functional depots for NO, which is a free radical with a short biological half-life’ for clarification.
2. Throughout results there is no information given as to when N6022 administration occurred in reference to the last ovalbumin challenge (e.g. 24 hours post). This information should be included in main text and all relevant figure legends.

Administration of N6022, IpBr + Alb, and PBS at 24 h prior to MCh challenge occurred on the same day as the last OVA airway challenge which was given on study day 22. In these instances, compounds were administered one hour prior to OVA. We have included this information in the Methods as well as in the appropriate Figure legends.

3. In the discussion (p.18-20) the authors do acknowledge some limitations of the study, namely that SNOs were not measured and that inhibition of GSNOR could not be directly detected. They should also state that collectively, their data does not rule out the possibility that the effects of GSNOR inhibition may be due to suppression of aldehyde clearance or some other function of GSNOR.

We have included a statement in the Discussion to address this additional limitation. Specifically, since we were not able to measure increased GSNO as a result of GSNOR inhibition in vivo we do not have definitive evidence that N6022 is inhibiting the oxidation of GSNO rather than the reduction of an aldehyde to cause the physiological effects. This being said, our findings do support the role of the GSNO reaction in N6022 mediated actions. In particular, decreased NFκB activity which is known to be controlled by NO, increased BALF nitrite, and increased plasma cGMP support the GSNO/NO mediated pathway.

Reviewer 3:

Major concerns

1. There is an unexplained absence of any actual direct airway mechanics measurements together with a lack of discussion of the well-established limitations of the “enhanced pause” (Penh) technique. The closest mention of this is in the Methods section where the authors note that the Penh “correlates with the measurement of airway resistance, impedance, and intrapleural pressure.” This would seem to be an important point given that even the title concludes the compound has bronchodilatory effects.

We did not conduct direct measurements of airway mechanics in this study as we preferred to first assess the actions of our novel compound in an asthma model in which the animals were not subjected to anesthesia, restraint, and intubation.

We do recognize the limitations of the Penh measurement and have included a statement as to the absence of direct measurement of airway mechanics as well as statements and references regarding the limitations
of the Penh technique within the Discussion.

2. The authors acknowledge an inability to detect SNOs in the BAL fluid of asthmatic mice, but some clarifications are needed. First, what was the detection limit and what assay(s) was/were used in the N30 labs and/or the contractor’s lab? Was there an attempt to measure GSNO or other SNOs in the unexposed mice? Please provide a reference for the statement concerning the dilution-induced dissociation of the inhibitors from the enzyme-substrate complex. Finally the authors refer to the inability of other investigators to detect GSNO in asthmatic BALF. A brief discussion of any difference in methods or other variables would be in order here. If the authors have any data on (G)SNO reductase activity in these mice or their BAL fluid using spiked GSNO, that would be supportive.

The detection limit of our SNO assay was 5 pmoles (100 μL injection volume of 50 nM lowest SNO standard). SNOs were assessed using tri-iodide reduction followed by ozone chemiluminescence detection with a nitric oxide analyzer (Sievers), and samples were analyzed with or without sulfanilamide treatment to remove the contaminating nitrite signal (Yang et al, 2003; Pinder et al, 2008). We attempted these measurements in both OVA sensitized and non-sensitized mice. We have included more detail on the assay employed and these references in the Discussion.

We have included a citation to one of our manuscripts in regard to the dilution-induced dissociation of the inhibitor from the enzyme-substrate complex (Green et al., 2012).

Que et al (2009) also mentioned the inability to detect GSNO in asthmatic BALF as we mentioned in the Discussion. The analytical method cited was measurement of SNOs in high molecular weight and low molecular weight (predominantly GSNO) BALF fractions. SNOs were measured using photolysis-chemiluminescence in the absence or presence of HgCl₂ to cleave thiol-bound NO (SNO). The method used by Que et al (2009) uses high energy, high temperature UV photolysis to liberate NO, which is prone to overestimation of SNO signal by contamination of NO released from nitrite (Gow et al 2007). The lowest GSNO standard was reported as 2 pmoles while total SNOs reported was 10-20 pmoles/mL (10-20 nM) in Que et al (2009), values which are close to our detection limit of 50 nM.

Our methods differ slightly from those used by Que et al; however, our findings and detection limits appear to be similar.
3. There appears to be a U-shaped relationship between the GSNO inhibitor dose and the Penh changes, eosinophilia, and changes in several of the biomarkers including IL-10, IL-12p70, IL-17A, in BALF and plasma fibrinogen. Please acknowledge the complex dose-response relationship and discuss as appropriate. Also, it would appear that the effects on BAL fluid fibrinogen and (at both doses) are significant but this is not so marked (error)?

The lack of further benefit observed as we approached or exceeded N6022 doses around 0.1 mg/kg was noted across several of the measured parameters. This observation or U-shaped relationship may be attributed to the complexities associated with GSNOR inhibition and the downstream pathways mediated by the GSNO/SNO pool. These complexities may involve the level of GSNO/SNOs produced, the nitrosation target, and the effect nitrosation, and whether the levels produced and downstream target(s) lead to a more beneficial or more detrimental effect on the measured parameter.

N30 is continuing to explore the mechanism of GSNOR inhibition in relation to complexities of NO biology.

N6022 significantly decreased BALF fibrinogen at both doses. We have corrected this error in Table 1.

Minor concerns

1. Please provide a reference for the statement concerning the dilution-induced dissociation of the inhibitors from the enzyme-substrate complex.

We have included our Green et al, 2012 reference in the Discussion.

2. BAL fluid nitrite, Figure 3, please show values from non-sensitized animals. Likewise, for Figure 4, cGMP.

We have revised Figure 3 which now includes BALF nitrite values for non-sensitized mice. BALF nitrite values are similar for non-sensitized mice and OVA-sensitized vehicle treated mice. Additional text has been included in the Results for this parameter.

Figure 4 depicts plasma cGMP from a time course study conducted with N6022. We do not have non-sensitized groups of mice from this study for comparison of the cGMP parameter. We also did not measure cGMP from non-sensitized mice from other studies reported in our manuscript.
3. Figure 6, consider going beyond calling this effect “sustained” and noting that it appears to strengthen over the time elapsed between inhibitor dosing and challenge

We have added text to describe that the effect of N6022 appears to strengthen over time as noted by greater effects when comparing responses at 1 h or 12 h to the later times of 24 h and beyond. The effects still appear to be sustained noted by similar responses at 24 h, 36 h, and 48 h after N6022 administration.

4. Bottom page 18 and top page 19. Regarding the statement that the findings suggest iNOS-derived NO is not necessarily responsible for SNO levels in the BALF, this statement requires some more explanation or evidence as currently stated.

We have included additional text to support the statement that iNOS-derived NO may not be response for BALF SNO levels. Particularly, we have shown that our GSNOR inhibitors attenuate iNOS expression in cellular models of inflammation.

5. Even putting aside the Buxco/Penh limitations, it would be more accurate to describe N6022 as preventing bronchoconstriction in response to methacholine in sensitized and, to a lesser extent, non-sensitized mice subjected to methacholine challenge, than to refer to bronchodilatory capacity

As recommended by Reviewer 1, we have included additional text within the discussion in regard to the limitations of the Penh measurement.

We prefer to leave in the term ‘bronchodilatory’ to describe the effect of N6022. The protocol for the OVA studies and the tracheal ring assay is more reflective of measuring prevention of MCh-induced bronchoconstriction. We do observe that N6022 causes a dose-dependent relaxation in rat tracheal rings that have been pre-contracted with MCh – we have included an additional panel in Figure 5 that depicts the ability of N6022 to relax pre-constricted tracheal rings as well as Methods and Results to describe this additional assay. Many of our other GSNOR inhibitors cause potent relaxation in the MCh-precontraction rat tracheal ring assay, achieving 100% relaxation at doses of 30 or 100 μM.

6. In the Figure 1 legend, please state that Figure 1D reports on non-sensitized mice.

The legend states this already.