EFFECT OF INHIBITION OF S-NITROSOGLUTATHIONE REDUCTASE ON THE NF-κB PATHWAY

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To my Son, Nick

Thank you for your encouragement, patience,

and support.

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12-HDDA	12-hydroxydodecanoic acid
12-ODDA	12-oxododecanoic
A549	human lung carcinoma epithelial cell line
ADH	Alcohol dehydrogenase
ADH1B	Alcohol dehydrogenase 1B; $\beta_2\beta_2$-ADH
ADH4	Alcohol dehydrogenase 4; π-ADH
ADH7	Alcohol dehydrogenase 7; σσ-ADH
ALF	airway lining fluid
Biotin-HPDP	N-[6-(Biotinamido)hexyl]-3´-(2´-pyridyldithio)propionamide
BOG	β-octyl glucoside
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
C1	3-[1-(4-acetylphenyl)-5-phenyl-1H-pyrrol-2-yl]propanoic acid
C2	5-chloro-3-{2-[(4-ethoxyphenol)(ethyl)amino]-2-oxoethyl}-1H-indole-2-carboxylic acid
C3	4-{[2-[(2-cyanobenzyl)thio]-4-oxothieno[3,2-d]pyrimidin-3(4H)-yl]methyl} benzoic acid
DMSO	dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FDH	Formaldehyde dehydrogenase

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	glutathione
GSNO	S-nitrosoglutathione
GSNOR	S-nitrosoglutathione redutase
GSSG	glutathione disulfide
HEN	Hepes, EDTA, neocupronine
HMGSH	hydroxymethylglutathione
HRP	horseradish peroxidase
IC_{50}	half maximal inhibitory concentration
ICAM	intercellular adhesion molecule
IκB	inhibitor kappa B
IKKβ	inhibitor kappa B kinase beta
iNOS	Inductible nitric oxide synthase
L-NAME	Nώ-Nitro-L-arginine methyl ester hydrochloride
MG-132	Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal
mICAM	transmembrane intercellular adhesion molecule
MMTS	S-methylmethanethiosulfonate
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide reduced
NF-κB	Nuclear factor-kappa B

NO	nitric oxide
PBS	phosphate buffered saline
pIκB	phosphorylated inhibitor kappa B
pIKKβ	phosphorylated inhibitor kappa B kinase beta
PVDF	Polyvinylidene difluoride
RAW 264.7	mouse Abelson murine leukemia virus transformed macrophage cells
RBC	red blood cells
SDS	sodium dodecyl sulfate
sICAM	soluble intercellular adhesion molecule
SNO	S-nitrosothiol
TBS	Tris buffered saline
TBS-T	Tris buffered saline with tween
TNFα	tumor necrosis factor alpha
WT	wild type
βME	beta mercaptoethanol

INTRODUCTION

I. Characterization of S-nitrosoglutathione Reductase

S-nitrosoglutathione reductase (GSNOR) also known as glutathione-dependent formaldehyde dehydrogenase (FDH), is a zinc-dependent dehydrogenase. It is a member of the alcohol dehydrogenase (ADH) family and is also called Class III alcohol dehydrogenase. The substrate specificity of GSNOR has been well studied (Holmquist and Vallee, 1991; Wagner *et al*., 1984). It oxidizes long chain alcohols to an aldehyde with the help of a molecule of NAD^+ (Figure 1). Alcohols with a carbon chain longer than four carbons and containing a carboxyl group at the opposite end are metabolized by GSNOR much more efficiently than ethanol (Sanghani *et al*., 2000; Wagner *et al*., 1984) an example is 12-hydroxydodecanoic acid (Figure 2A). As the carbon chain length of alcohol increases, the K_m of the alcohol decreases (Wagner *et al*., 1984).

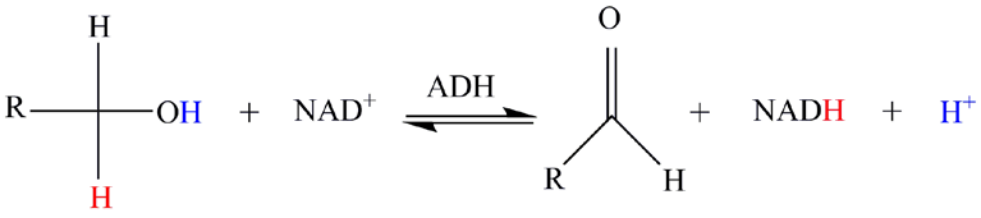
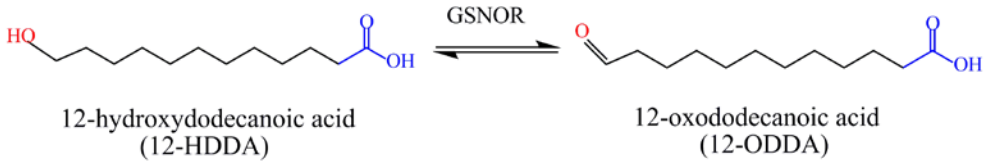


Figure 1. GSNOR Catalytic Reaction. GSNOR oxidizes an alcohol to aldehyde using NAD^+ as a coenzyme.

GSNOR was initially identified as FDH because of its role in the formaldehyde detoxification pathway. Hydroxymethylglutathione (HMGSH) is formed by the spontaneous reaction of formaldehyde and glutathione (GSH). FDH oxidizes HMGSH to S-formylglutathione (Figure 2B) with the help of NAD^+. S-formylglutathione is further converted to formic acid and glutathione enzymatically by S-formylglutathione hydrolase. Removal of formaldehyde from the cells protects the cells from its detrimental effects. Formaldehyde is detrimental to cells because it can modify proteins, damage membranes, and cause mutagenesis through DNA-protein cross-links (Barber and Donohue, 1998). Formaldehyde was commonly used as a fixative for cell and tissue cultures until it was declared a carcinogen.

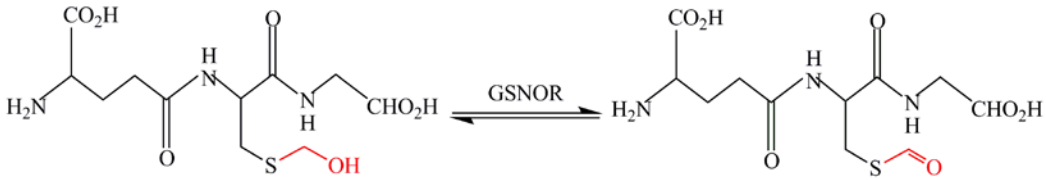
A)



12-hydroxydodecanoic acid (12-HDDA)

12-oxododecanoic acid (12-ODDA)

B)



HMGSH **S-formylglutathione**

Figure 2. Substrates for GSNOR. A) *In vitro* reaction of GSNOR: 12-HDDA is an example of a long chain primary alcohol with a carboxyl group which GSNOR oxidizes to an aldehyde. B) GSNOR plays a key role in the removal of formaldehyde from the body. Formaldehyde spontaneously reacts with GSH to form HMGSH, GSNOR catalyzes the reaction of HMGSH to S-formylglutathione which is hydrolyzed to glutathione and formic acid by S-formylglutathione hydrolase.

The crystal structure of GSNOR has been determined (Yang *et al*., 1997) and the enzyme exists as a homodimer in its native state. GSNOR contains a coenzyme binding site for NAD^+/NADH and a substrate binding site for GSNO or long chain alcohols (Figure 3).

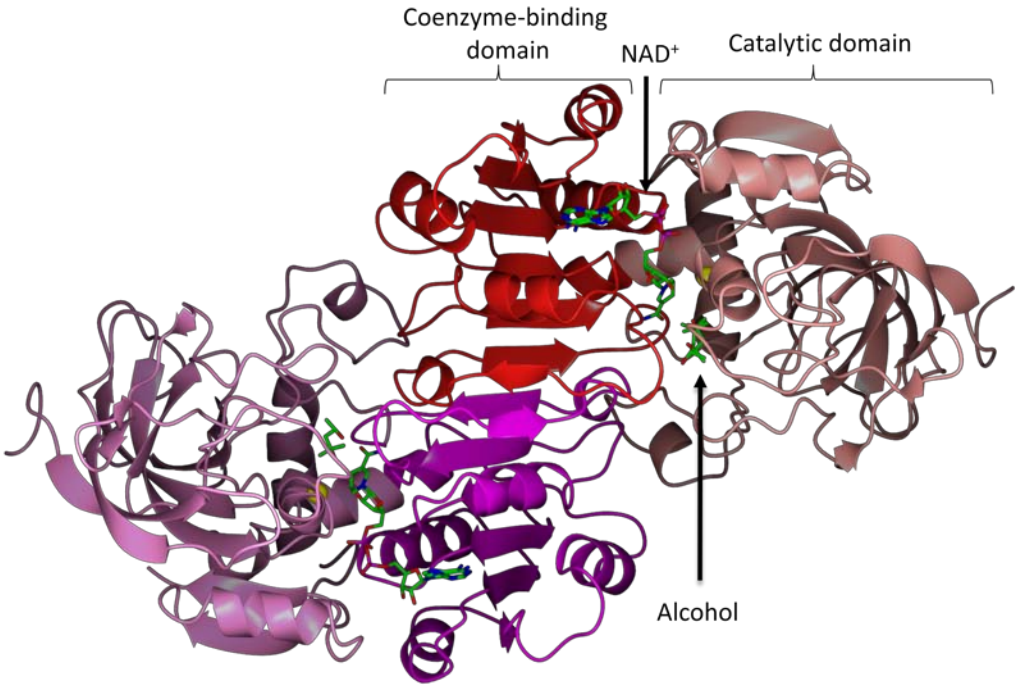


Figure 3. Structure of GSNOR Homodimer. Each GSNOR monomer contains a zinc ion, coenzyme binding site for NADH, and catalysis binding site for substrate.

GSNOR is ubiquitously expressed in tissues as compared to other ADHs (Kaiser *et al*., 1989; Sanghani *et al*., 2000; Estonius *et al*., 1996). One characteristic of ubiquitously expressed genes is the absence of a TATA box or a CAAT box in its promoter region (Hur and Edenberg, 1992), as is the case in GSNOR. GSNOR is a highly conserved enzyme and can be found in both prokaryotic and eukaryotic organisms. GSNOR has been maintained throughout evolution and is vital for NO homeostasis as a regulator for protein S-nitrosation through the reduction of GSNO (Liu *et al*., 2001). Although GSNOR is expressed

in all tissue, its activity levels are highest in the liver followed by kidney, heart, lung, spleen and thymus (Liu *et al*., 2004).

GSNOR was very well characterized as FDH until a report by Liu *et al*. identified NADH dependent GSNO-metabolizing enzyme as FDH by mass spectrophotometry (Liu *et al*., 2001). Subsequent studies revealed the GSNOR's oxidation rate for HMGSH is two to eight fold higher when GSNO is available than for HMGSH alone (Staab *et al*., 2008). The only S-nitrosothiol (SNO) substrate recognized by GSNOR is GSNO (Liu *et al*., 2004). A transnitrosation reaction transfers NO from nitrosylated proteins or S-nitrosothiols (RSNO) to glutathione to form S-nitrosoglutathione. This GSNO is finally converted to glutathione disulfide (GSSG) by a two step mechanism. First, GSNOR reduces GSNO to N-hydroxysulfenamide-glutathione (Fukuto *et al*., 2005) in the presence of NADH followed by non-enzymatic reaction of N-hydroxysulfenamide-glutathione and glutathione to glutathione disulfide and hydroxylamine (Figure 4). Cellular GSNO is a nitric oxide reservoir that can either transfer to or remove from the proteins a NO group. Reduction of GSNO by GSNOR depletes this reservoir and therefore indirectly regulates protein nitrosylation.

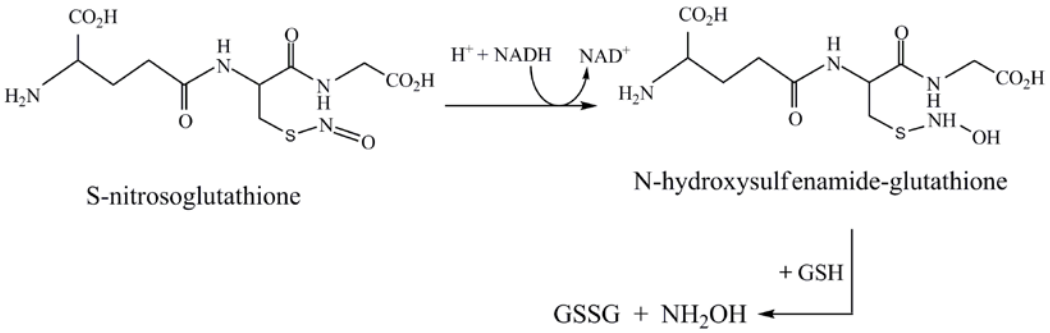


Figure 4. GSNOR Reduction of GSNO. The figure above shows the reduction of GSNO by GSNOR to a final product of GSSG and hydroxylamine.

II. Effects of the Inhibition of GSNOR

The role of GSNOR in NO metabolism is very well established by studies in GSNOR knockout mice (Liu *et al*., 2004). The effect of inhibition in GSNOR should result in an increase in RSNOs which is what was observed in GSNOR knockout mice (Liu *et al*., 2004). After treatment with lipopolysaccharide, RSNOs in $GSNOR^{-/-}$ mice increased 3.3-fold and 29-fold over wild-type (WT) mice at 24 and 48 hours, respectively. Of the RSNOs in the $GSNOR^{-/-}$ mice 90% are RSNOs with molecular weights >5000 (Liu *et al*., 2004). This increase in RSNOs effects vascular homeostasis, asthma, and cystic fibrosis. Hypotension was amplified in anesthetized $GSNOR^{-/-}$ mice over WT mice. The basal levels of SNOs in RBC's from $GSNOR^{-/-}$ mice were 2-fold higher, this amount would cause vasodilation in bioassays (Liu *et al.*, 2004). In the study by Que *et al.*, WT mice exposed to the allergen ovalbumin exhibited airway hyperresponsivity and were depleted of lung SNOs likely due to increased GSNOR activity seen in these mice.

In the same study, GSNOR$^{-/-}$ mice do not show airway hyperresponsivity upon exposure to ovalbumin (Que *et al*., 2005). Using a Human Airway Bioassay technique, Gaston *et al*. documented GSNO concentrations in asthma patients to be much lower than in control patients and correlated inversely to NO concentrations (Gaston *et al*., 1993). Elevated NO in patient's exhaled air, is one of the top symptoms in the diagnosis of asthma (Que *et al*., 2005). A depletion of GSNO in the airway lining fluid (ALF) of asthmatic patients also correlates with this symptom. In a clinical study, asthma patients have shown to have two times higher GSNOR activity than controls and depleted GSNO and SNOs in bronchoalveolar samples (Que *et al*., 2009). Asthma and cystic fibrosis patients have a decrease in GSNO concentration in the airway lining fluid. One treatment being investigated for cystic fibrosis patients is inhaling aerosolized GSNO (Foster *et al*., 2003; Zaman *et al*., 2006). GSNOR inhibitors which can increase the basal GSNO levels will be another potential therapy.

III. Effects on NF-κB Pathway

The NF-κB Pathway is regulated by series of positive and negative regulatory elements. Positive regulation causes phosphorylation of IKKβ, which in turn phosphorylates IκB. IκBα is an inhibitory molecule that sequesters NF-κB in the cytosol. Phosphorylation of IκBα targets it for ubiquination and proteasomal degradation releasing NF-κB (Figure 5). NF-κB then travels to the nucleus and initiates transcription of cytokines, chemokines, which includes genes

such as NOS, tumor necrosis factor alpha, (TNFα), intercellular adhesion molecule (ICAM), and self regulation. ICAM is a transmembrane protein (mICAM) or a soluble protein (sICAM) which is produced in epithelial cells and leukocytes (Hayden *et al*., 2006; Whiteman and Spiteri, 2008). ICAM adheres the leukocytes to the affected endothelial cells. Leukocytes are the defense mechanism of the body and will migrate into the infected tissue (Hayden *et al*., 2006). Mice that have inadequate amounts of p65 NF-κB lack the ability to adhere leukocytes to the epithelial cells which slows the immune response (Hayden *et al*., 2006).

The role of nitric oxide in regulation of NF-κB pathway is reviewed by Bove and van der Vliet (Bove and van der Vliet, 2006). IKKβ has been shown to be a direct target for SNO modification resulting in decreased IKKβ activity causing inhibition of NF-κB dependent transcription (Reynaert *et al*., 2004). Asthma is the overstimulated inflammatory pathway in response to allergens entering the respiratory system. The fact that asthma patients have decreased amounts of GSNO could result from overproduction of GSNOR causing an imbalance of NO and S-nitrosylated proteins; therefore activating the NF-κB pathway and consequently the immune response. Inhibiting GSNOR would prevent the rapid removal of NO from S-nitrosylated proteins including IKKβ and therefore could impede the NF-κB pathway, slowing the immune response in asthma patients (Figure 5).

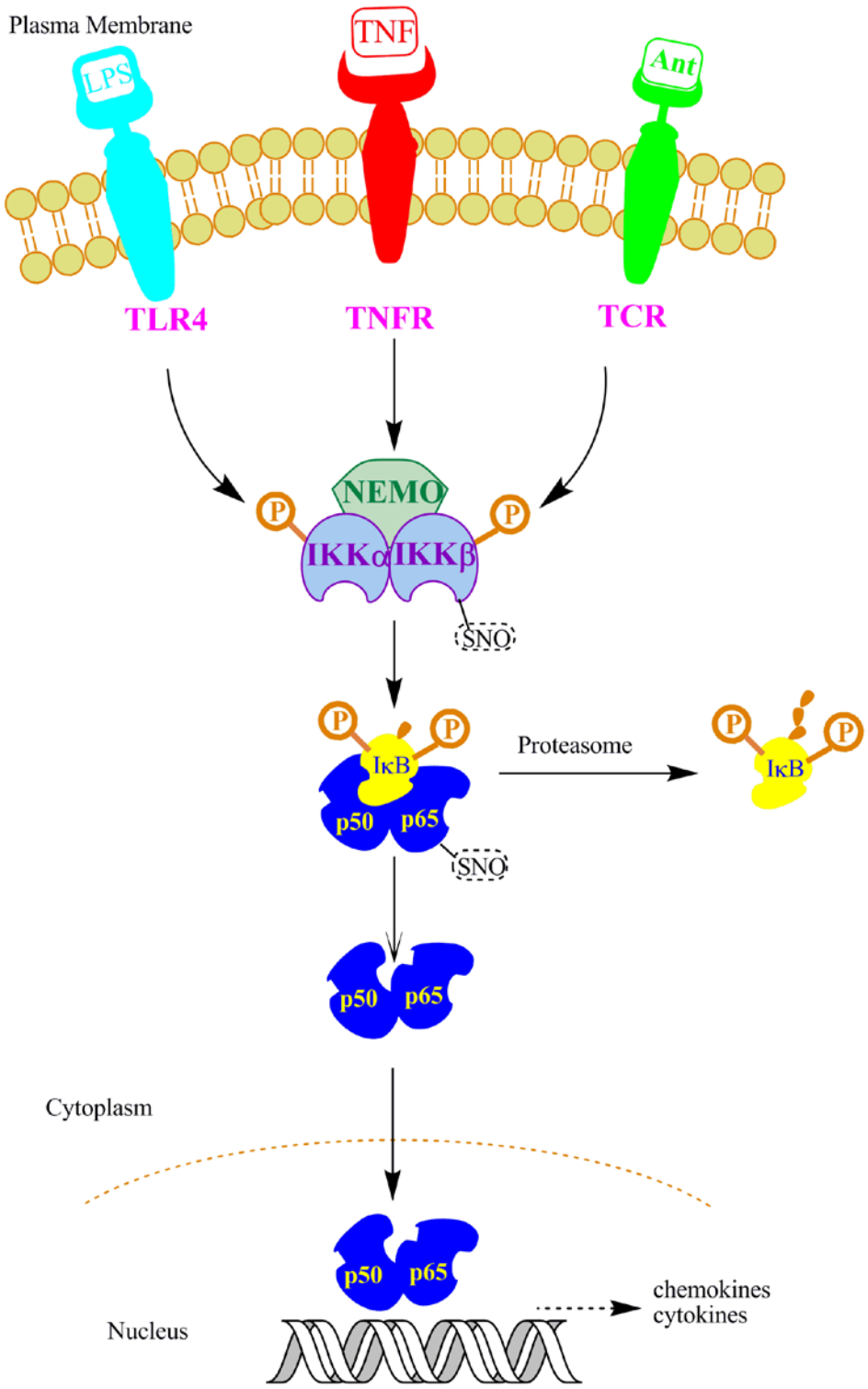


Figure 5. NF-κB Pathway. The NF-κB is a key transcription factor for the transcription of cytokines and chemokines.

IV. Small Molecule Inhibitors of GSNOR

High-throughput screening was performed using ChemDiv Inc's small molecule library of 60,000 compounds for inhibition of GSNOR activity in Chemical Genomics Core facility at Indiana University. Recombinant GSNOR was expressed in *E. coli* and purified using a previously described method (Sanghani *et al*., 2000). In high-throughput screening GSNOR activity was determined using 384 well plates with substrate octanol and cofactor NAD^+. The production rate of NADH absorbance at 340 nm was monitored. Potential compounds were selected based on the ability to inhibit the activity of GSNOR and retested in the laboratory using an IC_{50} *in vitro* assay. If the compound showed a 100 fold lower IC_{50} than the known inhibitor dodecanoic acid concentration in the IC_{50} *in vitro* test (Table 1) they were selected for further studies. The small molecules were then tested for the inhibition of other ADHs, allowing selection of compounds that exclusively inhibit GSNOR (Table 2).

Compound Number	% inhibition		IC_{50} at pH 7.5 μM
	pH 10	pH 7.5	
Dodecanoic acid	4	19	212
C1	78	93	1.3
C2	55	91	2.4
C3	75	95	1.1

Table 1. *In vitro* Percent Inhibition and IC_{50} of C1, C2, C3. The percent inhibition was determined using 2 conditions. Inhibition studies at pH 10 were performed in 0.1 M sodium glycine (pH 10), 1 mM octanol, 1 mM NAD^+, 0.1 mM EDTA and 50 μM inhibitor. Inhibition studies at pH 7.5 were performed in 50 mM potassium phosphate pH 7.5 containing 15 μM NADH, 10 μM GSNO, 0.1 mM EDTA and 50 μM inhibitor.

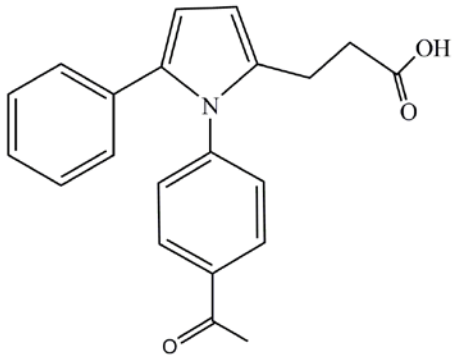
Enzyme	% Inhibition		
	C1	C2	C3
GSNOR	77	71	73
ADH1B	5	0	5
ADH7	4	13	8
ADH4	6	1	3

Table 2. Compound Inhibition Comparison of ADHs. Inhibition studies were performed in presence or absence of 5 μM inhibitor at 25ºC in 50 mM potassium phosphate pH 7.5 containing 0.1 mM EDTA. The enzyme activity was measured by following the changes in absorbance at 340 nm. The values show the percent reduction in the enzyme activity caused by the inhibitor. The standard errors are below 15% of the averages shown, except when the inhibition was below 20%. Studies with ADH1B ($\beta_2\beta_2$-ADH), ADH7(σσ-ADH), ADH4 (π- ADH) were performed in 0.05 % DMSO. Studies with GSNOR were performed in presence of 1 % DMSO.

From the *in vitro* IC_{50} and inhibition studies, three candidates, C1, C2, and C3, (Figure 6A-C) were selected and assessed *ex vivo* using RAW 264.7 macrophage cells and A549 human carcinoma lung epithelial cells (Sanghani *et al*., 2009). Of these three inhibitors all experiments were completed using C3. Inhibitor C2 experiments were performed during cytotoxicity, nitrosylation, and C1was only analyzed early in the studies, *ie*. cytotoxicity experiments. Initially C3 showed higher level of protein nitrosolyation in cells making C3 our first choice. Some experiments with C2 were included for comparison to C3.

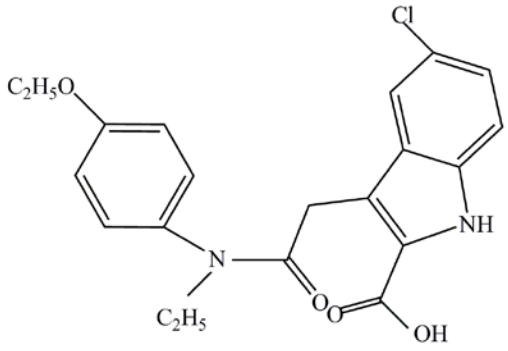
Very little data for C1 was gathered because the results did not show sufficient nitrosylation in cell lysates compared to C2 and C3. During ongoing experiments C3 seemed to exhibit more results, *ie*. detection of more nitrosolyation in cell lysates, more defined IκB experiments than C2 and C3.

C1



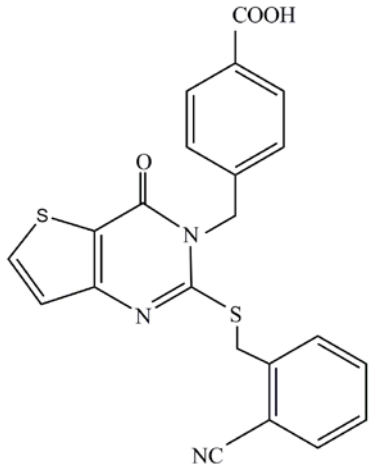
3-[1-(4-acetylphenyl)-5-phenyl-1H- pyrrol-2-yl]propanoic acid

C2



5-chloro-3-{2-[(4-ethoxyphenyl)(ethyl)amino]-2-oxoethyl}-1H-indole-2-carboxylic acid

C3



4-{[2-[(2-cyanobenzyl)thio]-4-oxothieno[3,2-d]pyrimidin-3(4H)-yl]methyl}benzoic acid

Figure 6. Inhibitors of GSNOR. Structures of the GSNOR inhibitors C1; C2; C3.

V. Biotechnology

Several biotechniques were utilized to obtain the data demonstrating the effects of inhibition of GSNOR on the NF-κB Pathway. Western blot is a critical technique and can be used for protein identification and semi-quantitation. Therefore selection and dilution of antibody are essential for cell culture techniques such as sterility, plating and selection of cells, are also crucial. ELISA and spectrophotometry were also utilized.

MATERIALS AND METHODS

I. Cytotoxicity of GSNOR Inhibitors

Cell Viability

Cell viability was determined using Promega CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega One Assay). The Promega One Assay is a colormetric method determining viable cells by production of formazan which absorbs at 490 nm. The production of formazan is directly proportional to the number of live cells. Human lung adenocarcinoma epithelial cell line, A549 (ATCC) was cultured in ATCC F-12K media containing 10% heat inactivated FBS and 1% pen/strep, at 37°C and in 5% CO_2. On day 1, 1500 A549 cells were plated in 100 µl medium per well of a 96-well tissue culture plate. The next day cells were treated with 100 µl of inhibitor C3 diluted in medium, for a final concentration 0, 10, 30, 50, 80, 110, 150 µM and incubated for 4 hours at 37°C. TNFα was added to the cells to a final concentration of 10 ng/ml and incubated for 5 days. The medium was replaced with 120 µl of fresh media containing 20 µl of the Promega One Assay Reagent. The cells were incubated again at 37 °C for 1hour and then the absorbance was determined at 490 nm using a Molecular Devices SpectraMax 190 microplate reader.

A second experiment was completed with minor changes to determine the cell viability for inhibitors C1, C2, C3. The total time of treatment for this

experiment was 10 hours. On day 1, 1500 A549 cells were plated in 100 μl medium per well of a 96-well tissue culture plate. The next day cells were treated with 100 μl of inhibitor, C1, C2, or C3 diluted in medium, for a final concentration of 0, 10, 30, or 75 μM and incubated for 4 hours at 37°C. TNFα was added to the cells to a final concentration of 10 ng/ml and incubated for an additional 6 hours at 37°C. Measurements were made using Promega One Assay reagent as described above.

Cell Proliferation

The cell proliferation was determined using Bromodeoxyuridine Cell Proliferation Assay kit (Calbiochem). This assay uses Bromodeoxyuridine (BrdU) as a nucleoside to replace deoxythymidine in newly synthesized DNA produced in the S-phase of the cell cycle. A549 cells were plated in 96-well plates at 1500 or 4000 cells per well in 100 μl media and incubated overnight. The cells were treated with 0, 0.3, 1, 3, 10, 30, 50, or 100 μM of C2 or C3 and incubated for 24 or 48 hours at 37°C. The relative amount of BrdU incorporated is then determined by an ELISA test using anti-BrdU monoclonal antibody and a secondary antibody conjugated with HRP. The HRP product is proportional to the incorporated BrdU.

II. Identification of S-nitrosylated Proteins

On day 1, twelve 100 mm dishes were plated with 3 x 10^6 Raw 264.7 cells per dish in 10 ml of DMEM F-12 media (ATCC) + 10% heat inactivated fetal

bovine serum (Atlanta Biologicals) + 1% penicillin/streptomycin (Invitrogen). On day 2, the medium in the dishes was replaced with 9 ml of fresh DMEM medium prior to treatment. Cells were treated according to Table 3 to obtain a final concentration of, 33 µM for C3 and 1.1 µM for L-NAME. Cells were incubated at 37°C and 5% CO_2 with these compounds for 2, 4, 8 or 24 hours then lysed with HEN lysis buffer (250 mM HEPES pH 7.7, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM Neocupronine, 1% Nonidet P-40 (Sigma) and 10 mM S-methyl methanethiosulfonate (MMTS)). Because of the photosensitivity of the SNOs', all handling of samples after treatment was completed under special yellow lights.

Plate #	Treatment	Time
1, 7	DMSO	4hr
2, 8	C3 33 µM	2 hr
3, 9	C3 33 µM	4hr
4, 10	C3 33 µM + 1.1 µM L-NAME simultaneous treatment	4hr
5, 11	C3 33 µM	8 hr
6, 12	C3 33 µM	24 hr

Table 3. Treatments of Raw 264.7 cells with C3.

Lysate Preparation

After treatment, in controlled lighting, the medium was removed and the cells were washed twice with 10 ml of phosphate buffer saline (PBS). After removal of the PBS, cells were scraped off in 500 µl of freshly made HEN lysis buffer. Lysate was placed in a microfuge tube and vortexed. Samples were then centrifuged at 16,100 x *g* for 10 minutes at room temperature. The supernatant (lysate) was removed, snap frozen using liquid nitrogen and stored at ¯20°C.

Protein concentration of the lysates was determined by Bio-Rad Protein assay using 2.5 µl of lysate per assay and bovine serum albumin (BSA) as standard.

Biotin Switch Assay

The lysates from the samples above were analyzed for S-nitrosothiols using the Biotin Switch Assay described by Jaffrey *et al* with a few modifications (Jaffrey and Snyder, 2001; Wang *et al*., 2008; Zhang *et al*., 2005). The product of this assay switches the S-nitrosothiols with biotin. To 200 µg of total protein from above lysates was added, 80 µl of 25% sodium dodecyl sulfate (SDS), 6 µl of 2 M MMTS (final concentration 10-15 mM MMTS) and Hen buffer to a total volume of 1 ml. Samples were incubated in a 50°C water bath for 25 minutes with occasional vortexing.

Five ml spin columns were prepared with Sephadex G25 resin equilibrated in chelexed PBS containing 1% SDS and 0.1 mM EDTA and centrifuged at 1000 x *g* for 2 minutes to pre-pack the columns. The samples were loaded onto the columns and centrifuged at 1000 x *g* for 2 minutes. The eluant was sequentially passed through two additional spin columns. The volume of the final eluant was measured and divided equally into 2 microfuge tubes, labeled ***A*** and ***B*** for the Biotin-HPDP labeling. Two hundred µl of 4 mM biotin-HPDP + 250 µl of dilution buffer (100 mM HEPES pH 7.7, 2.5 mM EDTA, 0.25 mM Neocuproine) were added to tubes labeled A. Two hundred µl of 4 mM Biotin-HPDP + 48 µl of 500 mM ascorbic acid + 200 µl regular water + 25 µl saturated cuprous chloride (CuCl) were added to tubes labeled B for biotin labeling. Both sets of tubes were kept in the dark at 25ºC for 3 hours. Five ml spin columns containing Sephadex G25 resin, equilibrated with 25 mM HEPES pH 7.7, 100 mM NaCl, 1 mM EDTA

and 0.5% β-octyl glucoside (BOG) were pre-packed by centrifugation at 1000 x *g* for 2 minutes. Samples were loaded onto the columns and centrifuged at 1000 x *g* for 2 minutes. The eluant was then sequentially placed on two additional columns to remove free biotin. Protein concentration was determined using 25 µl of each sample, using BCA Protein Assay (Pierce). Five µg of each sample was transferred to a new tube and β-ME free Lamelli buffer was added in preparation for the SDS-PAGE/Western blot assay.

Western Blot for Biotin Detection.

The biotin labeled lysate was analyzed by Western blot to establish the nitrosylation effect of the GSNOR inhibitors over selected time points. Five µg of protein from samples described above was boiled for 5 minutes and centrifuged at 16100 x *g* for 5 minutes. One µg of protein was loaded on a precast 10% acrylamide Tris-HCl gel (BioRad). Gel was electrophoresed for 2 hours at 120V. The proteins were immediately transferred to a PVDF membrane using the Bio-Rad Criterion Transfer Blotting apparatus with Tris/Glycine buffer pH 8.3 containing 10% methanol for 45 minutes at 100 V or overnight at 30V at 4°C. The blot was blocked with 5% milk in Tris buffered saline containing 0.1% Tween 20 (TBS-T). The blot was rinsed with TBS-T and probed with an anti-biotin HRP conjugated antibody (Sigma) for 1 hour at room temperature (RT). The blot was rinsed 3 x 10 minutes with TBS-T and developed using ECL Plus chemiluminescence kit (GE Healthcare).

Streptavidin Precipitation

The remaining lysate of the samples which were used in the Biotin Switch assay was subjected to acetone precipitation. The samples were centrifuged and the protein pellet was dissolved in 30 µl of 8 M guanidine and 170 µl of 10 mM potassium phosphate (KP_i) pH 8.0. Protein concentration was determined by the Bio-Rad Protein Assay using BSA as standard.

Twenty µl of streptavidin agarose resin (Pierce, Rockford, IL) equilibrated in 25 mM HEPES pH 7.7, 100 mM NaCl, 1 mM EDTA, and 1% BOG (neutralization buffer) were added to equal amounts of protein for all samples. The samples were diluted to 1 ml with neutralization buffer and incubated for 4 hours at 4°C with shaking. Samples/beads were centrifuged at 100 x *g* for 30 seconds, supernatant discarded, the beads were then washed 5 x 1 ml of washing buffer (25 mM HEPES pH 7.7, 600 mM NaCl, 1 mM EDTA, 1% BOG). The samples were incubated with Lamelli buffer containing 500 mM β-mercaptoethanol (βME) for 2 hours at 40°C with shaking. Samples were boiled for 5 minutes, centrifuged at 9300 x *g* for 5 minutes, and all of the supernatant was loaded on a Precast 10% acrylamide Tris-HCl gel (Bio-Rad).

Western Blot of Streptavidin Precipitated Proteins

The SDS-PAGE gel was immediately transferred to a PVDF membrane using the Criterion Transfer Blotting apparatus (Bio-Rad) with Tris/Glycine buffer pH 8.3 with 10% methanol for 45 minutes at 100 V or overnight at 30 V. The blot was blocked with 5% milk in Tris buffered saline and 0.1% Tween 20 (TBS-T).

The blot was rinsed with TBS-T and probed with the primary antibody IKKβ (Cell Signaling Technology) in 5% BSA-TBS-T overnight at 4°C. The blot was rinsed 3 x 10 minutes with TBS-T and incubated with HRP conjugated secondary antibody for 1 hour at room temperature, rinsed again 3 x 10 minutes with TBS-T. Blot bound HRP was detected using ECL Plus chemiluminescence kit (GE Healthcare).

III. Effect of Inhibitor C3 on NF-κB Pathway Proteins

Cell Treatment and Lysate Preparation

Two hundred thousand A549 cells, (ATCC) were plated in 2 ml of F-12K Media containing 10% heat inactivated FBS and 1% pen/strep into 35 mm dishes. Next day, the medium was replaced with 2 ml of fresh medium prior to treatment. Samples were incubated with inhibitor C3 at concentrations of 0, 30, 50, or 100 μM for 4 hours at 37°C then incubated with 10 ng/ml of the cytokine, TNFα, (Invitrogen) for 5 minutes at 37°C. The medium was quickly removed and the cells were rinsed with cold PBS. The cells were quenched with Lamelli buffer containing 50 mM NaF then extracted by scraping the dish clean. Lysate was vortexed, centrifuged briefly, boiled for 5 minutes, centrifuged at 16,100 x *g* for 5 minutes. Equivalent volumes of sample supernatants were loaded onto a precast 10% polyacrylamide Tris-HCl gel. A second experiment was completed with pre-treatment of C3 at concentrations 0, 50 and 100 μM for 4 hours. At the 3 hour mark MG-132, a known proteasome inhibitor, was added to a final concentration

of 40 μM). Then after 4 hours total, TNFα was added to specific samples for 5 minutes at 10 ng/ml.

Western Blot

The gel was transferred to a PVDF membrane and the membrane was blocked with 5% milk in TBS-T. The blot was then probed overnight at 4°C with primary antibodies phospho-IκBα, IκBα, phospho-IKKα/β, and GAPDH (Cell Signaling Technology and Santa Cruz Biotechnology) in 5% BSA in TBS-T. Appropriate secondary antibodies conjugated with HRP were incubated for 1 hour at RT. HRP was detected using ECL Plus chemiluminescence kit (GE Healthcare).

IV. Proteins Affected by Inhibition of NF-κB Pathway

Cell Extract Preparation

A549 cells were plated at 50,000 cells per well of a 24 well plate in 1 ml of F12K Medium containing 10% heat inactivated FBS and 1% pen/strep on the day before treatment. The cells were treated with 0, 10, 20, 30 and 50 μM of C3 for 4 hours at 37°C. TNFα was added to the appropriate wells for a final concentration of 10 ng/ml and incubated for 6 hours at 37°C. The reaction was quenched using Lamelli buffer (no βME). Cells were scraped from wells and placed in microfuge tubes. Cell extracts were frozen overnight at ¯20°C. Protein concentration was determined using BCA Protein Assay (Pierce). Two μl of βME was added to each sample. Samples were vortexed, boiled for 5 minutes centrifuged at 16,100 x *g* for

5 minutes. Equivalent amounts of supernatant (30 μl) were loaded onto a precast 10% acrylamide Tris-HCl gel.

Western Blot

Gel was immediately transferred as written above. The blot was probed with primary antibodies for ICAM, iNOS, COX2 and GAPDH (Santa Cruz Biotechnology) by incubating for 1-3 hours at RT. The blot was rinsed with TBS-T followed by incubation with the appropriate HRP conjugated antibody for 1 hour at RT. HRP signal on the blot was detected using ECL Plus chemiluminescence kit (GE Healthcare).

RESULTS

I. Cytotoxicity of GSNOR Inhibitors

Two methods were used to analyze the cytotoxicity of the inhibitors. The first method Promega One Assay, determines the number of live cells by detecting the amount of NADPH or NADH produced and present in the cytosol of the cell. The NADH or NADPH of live cells is the electron donor which converts MTS tetrazolium to formazan. The final product, formazan, is located in the cytoplasm, plasma membranes and other cellular compartments with a minor fraction in the mitochondria (Bernas and Dobrucki, 2002). The second assay, BrdU Assay measures the cells that can replicate its DNA in the S-phase of the cell cycle. The two types of assays were completed to determine the effect of the inhibitor on the cells. Is the inhibitor detrimental to the cells in 10 hours, or over longer periods of time, 48 hours. Both assays were used to determine how the inhibitor was affecting the cell *ie*. stopping replication or causing apoptosis.

Cell Proliferation

Cell Proliferation was evaluated using the BrdU Assay to determine the cytotoxicity of the inhibitors. The IC_{50} of C2 and C3 for the inhibition of A549 cells was found to be 60 μM $\pm$ 3 and 20 μM $\pm$ 5 respectively (Figure 7 and 8). These data suggests that cell proliferation is interrupted during long continuous exposure to the inhibitor (48 hours). This experiment also shows inhibitor C2 is 3 fold less cytotoxic than C3.

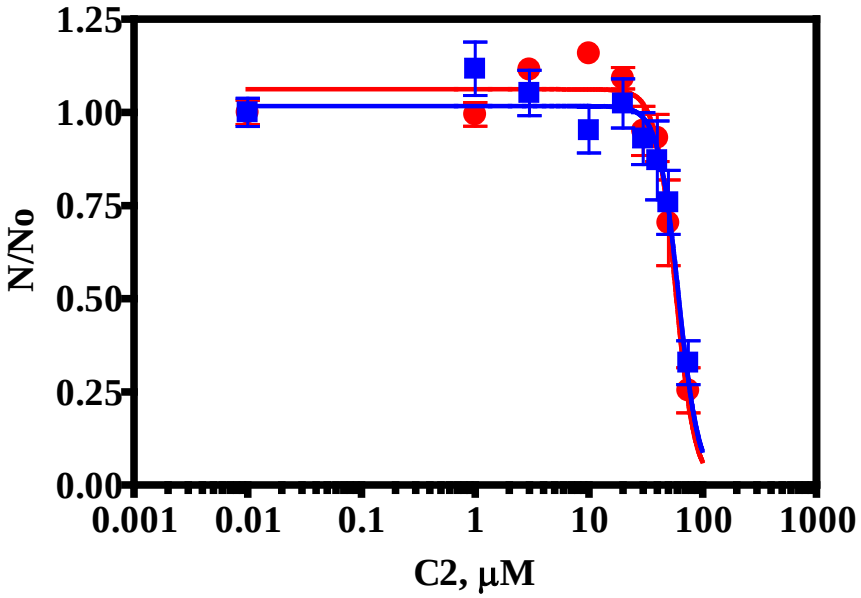


Figure 7. Inhibition of A549 cells by C2 using BrdU Incorporation. The IC_{50} of duplicate assays was analyzed using the BrdU Cell Proliferation Assay kit (Calbiochem). The IC_{50} of the inhibitory compound C2 in A549 cells after incubating with the treatment for 48 hours was 60 µM + 3 (n=12). IC_{50} was calculated using equation $Y=Bottom + (TOP-Bottom)/(1+(X/IC50)^{h})$ in GraphPad Prism 4.

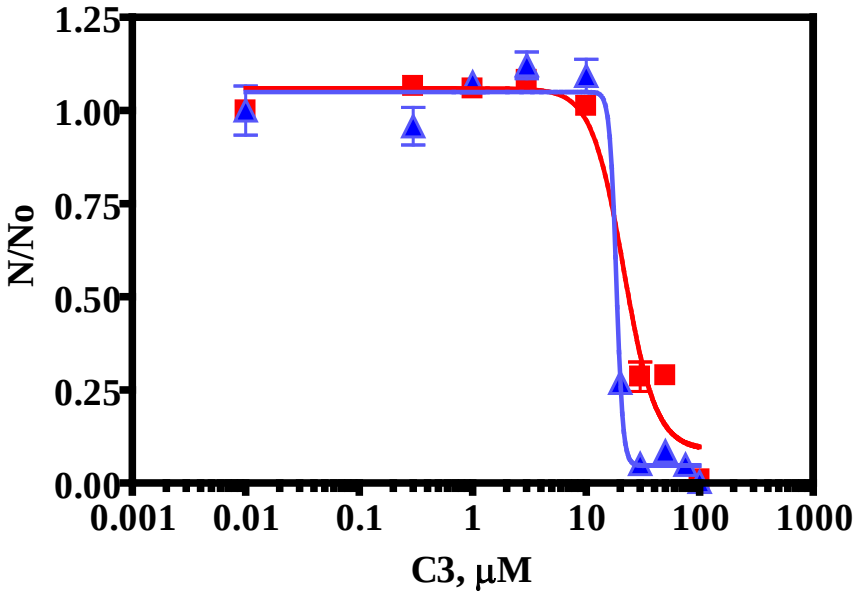


Figure 8. Inhibition of A549 cells by C3 using BrdU Incorporation. The IC_{50} of duplicate assays was analyzed using the BrdU Cell Proliferation Assay kit (Calbiochem). The IC_{50} of the inhibitor C3 was 20 µM $\pm$ 5 (n=12), in A549 cells after 48 hours incubation with the compound.

Cell Viability

The cell viability for inhibitor C3 was also determined using the Promega One Assay after 5 days of incubation with C3 in the presence or absence of TNFα. The IC_{50} of C3 without TNFα was 42 µM $\pm$ 12 and 40 µM $\pm$ 11 with TNFα (Figure 9).

The BrdU Assay of inhibitor C3 gives an IC_{50} two times lower than the Promega One Assay (20µM vs 40 µM). The difference of these results is attributed to the method of detection of cell cytotoxicity in the assays. The BrdU

Assay measures the cell division by analyzing the new DNA synthesized while Promega One Assay analyzes the NADH production of the cell. Live cells which are functional can produce NADH but may not be able to replicate.

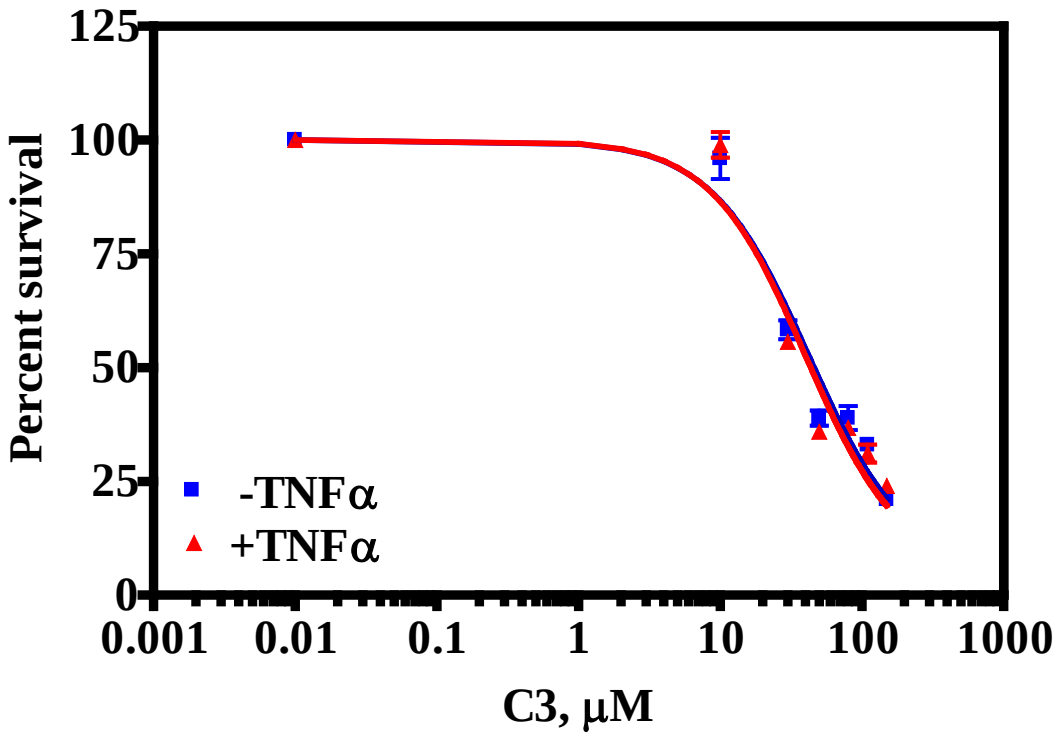


Figure 9. Inhibition of A549 cells by C3 with and without TNFα. The IC_{50} was analyzed using Promega CellTiter 96 AQ_{ueous} One Solution Cell Proliferation. The IC_{50} of the inhibitor C3 was 41 μM ± 11 (n=8), in A549 cells after 5 days of incubation the compound and in the prescence (42 μM +12) or absence (40 μM + 11) of TNFα.

After determining the IC_{50} of the compounds, a cell viability experiment was designed to establish the condition of the A549 cells treated with inhibitors C1, C2, and C3, under experimental conditions. The experimental conditions

were 4 hours of preincubation with cells and compound and then 6 additional hours with TNFα. Most of the data collected to study the NF-κB pathway was conducted within these limits. Inhibitors C1, C2, and C3 show a minimal change when normalized to untreated cells with a cell viability above 90% for the maximum inhibitor concentration tested, 75 µM (Figure 10). This cell viability of 90% provides confidence that the cells can withstand exposure to inhibitors C1, C2 and C3 when treated with TNFα for short time periods. This information also provides confidence in the results of the data used to define the inhibitor C3's effect on the NF-κB pathway.

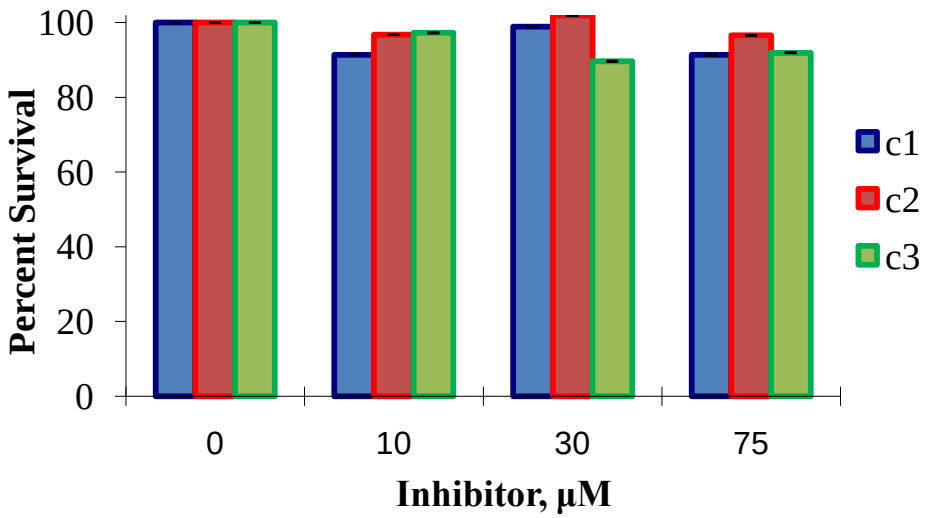


Figure 10. Cell Viability after 10 hours of Incubation with Inhibitors. A549 cells were treated with C1, C2, or C3 at indicated concentrations for 10 hours (4 hours preincubation and additional 6 hours after TNFα was added) and the viability was determined using the Promega CellTiter 96 AQ_{ueous} One Solution Reagent.

II. Identification of S-nitrosylated Proteins

The effects of GSNOR inhibition on the S-nitrosylation of cellular proteins were examined using the Biotin Switch assay. The nitrosylated proteins were labeled with biotin for analysis using the Biotin Switch Assay. The Biotin Switch Assay is necessary because S-nitrosothiol groups are very unstable and easily lost during sample treatment. This assay is a multiple step assay which removes the S-nitrosothiol group linked to the cysteines and replaces it with biotin, see Figure 11. The detection of the biotin in the biotin + ascorbate samples in the following experiments would be equivalent to the analyzing S-nitrosothiol.

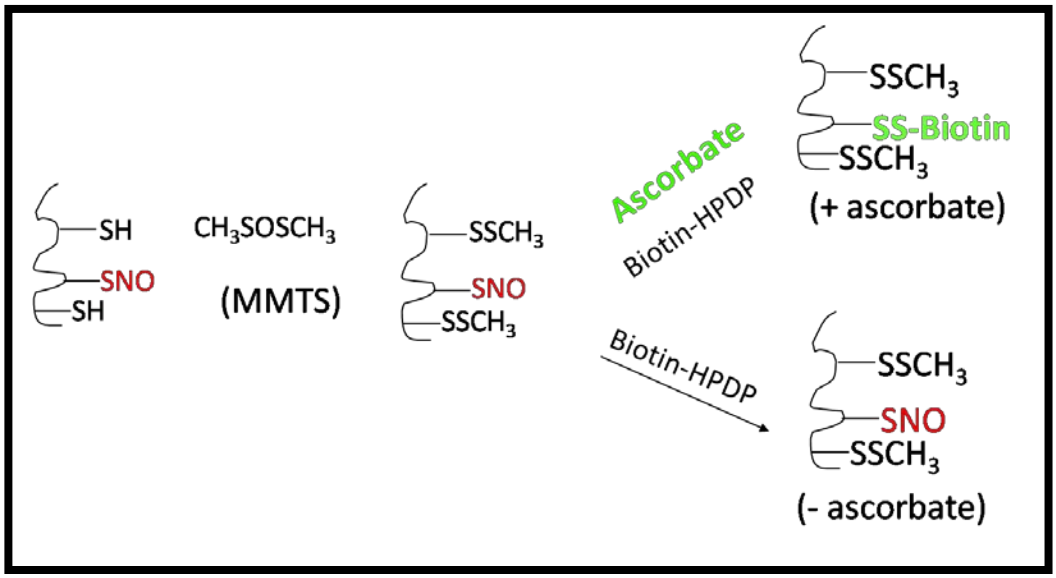
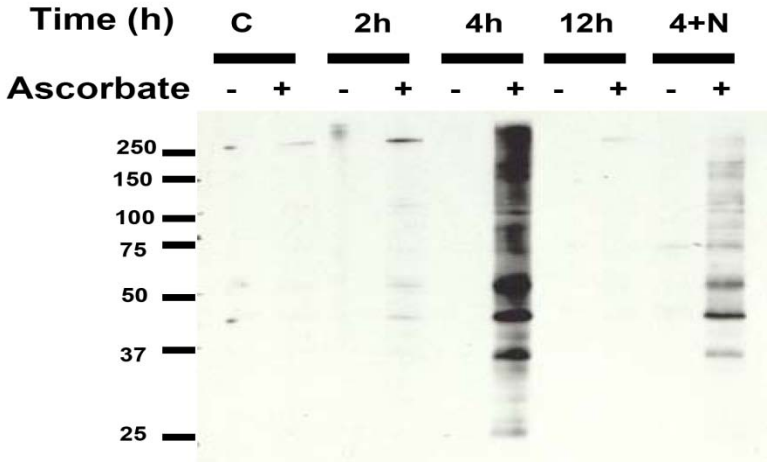


Figure 11. Schematics of the Biotin Switch Assay. Methyl methanethiosulfonate (MMTS) is first added to the lysate to block the free thiols (SH) of proteins with a methyl group. After free MMTS is removed the sample is split and N-[6-(Biotinamido)hexyl]-3´-(2´-pyridyldithio)propionamide (biotin-HPDP) plus or minus ascorbate (ascorbate, a weak reduction agent, can specificly reduce S-nitrosothiols to thiols) is added to the sample. These free thiols generated from SNO are then crosslinked with biotin using biotin-HPDP. The free biotin-HPDP is removed by gel filtration.

Raw 264.7 cells were treated with C2 or C3 for varied lengths of time, alone or in presence of nitric oxide synthase inhibitor, L-NAME (Sigma). L-NAME is an analog of arginine that inhibits the production of NO by inhibiting nitric oxide synthase. The Western blots in Figure 12 shows that nitrosylated proteins accumulate with time in cells treated with compound C2 and C3. The nitrosylation of intracellular proteins is evident following a 2 hour incubation with C3 and peaks at 8 hours before coming down to basal levels after 24 hours. Treatment with C2, nitrosylation peaked at 4 hours and back to basal levels at 12

hours. The amount of SNOs is similar in untreated cells and those treated with both C2 and L-NAME or C3 and L-NAME simultaneously. The L-NAME data illustrate the need of an active nitric oxide synthase to increase the accumulation of nitrosylated proteins and the inhibitor data suggest that inhibiting GSNOR increases the accumulation of large molecular weight SNOs in the cell.

A)



B)

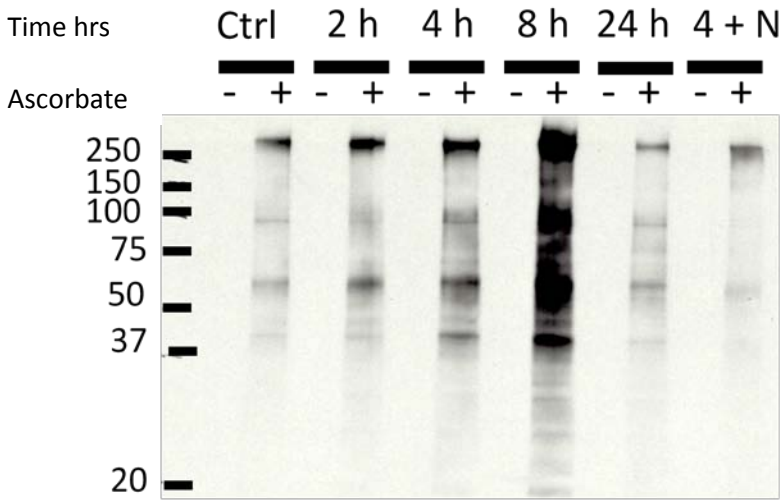


Figure 12. Nitrosylated Proteins after Treatment with C2 or C3. Cells were treated with 33 μM C2 or C3 for varied lengths of time (0, 2, 4, 8, 12 or 24 hours) alone or in combination with 1.1 mM L-NAME for 4 hours (lane 4+N). At indicated times, the cells were quenched and the lysate was analyzed for S-nitrosothiol content by the biotin switch assay. Equal amounts of proteins were loaded in each lane and the degree of biotinylation (and hence S-nitrosylation) determined using an anti-biotin antibody. A) A549 cells treated with C2; B) RAW 264.7 cells treated with C3.

With the results of S-nitrosolyated proteins increasing with time in cells treated with C2 or C3, the next step was to examine the effects of GSNOR inhibition on proteins reported to be affected by nitrosylating agents like S-nitrosoglutathione or S-nitrosocysteine. Several proteins in the NF-κB signaling pathway have been reported to be regulated by S-nitrosylation (Reynaert *et al*., 2004). Inhibitor Kappa B Kinase beta (IKKβ) was reported to be the most upstream nitrosylated protein in the NF-κB activation pathway. The effect of GSNOR inhibition on the S-nitrosylation of IKKβ was studied in RAW 264.7 cells using the biotin switch assay.

The biotinylated proteins were isolated using streptavidin agarose and subjected to Western blotting for the detection of specific proteins. The Western blot in Figure 13 shows IKKβ to be nitrosylated in RAW 264.7 cells following the inhibition of GSNOR with C3. The extent of S-nitrosylation of IKKβ also increased with the length of exposure of cells to C3. Concomitant treatment of cells with L-NAME and C3 did not change the S-nitrosylation level of IKKβ. This suggests the inhibition of GSNOR with C3 increases the accumulation of nitrosylated IKKβ over time but does not nitrosylate IKKβ.

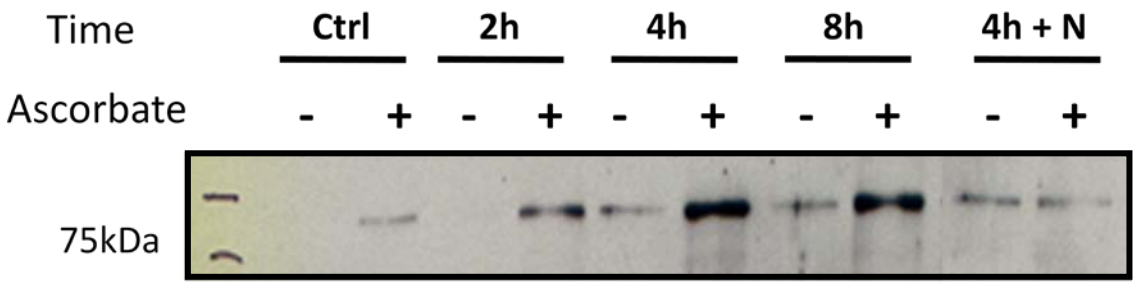


Figure 13. S-nitrosylated IKKβ of Treated Samples Following Treatment with C3. RAW 264.7 cells were treated with C3 for 0, 2, 4, 8 hours and 4 hours + L-NAME. After the S-nitrosylated proteins were labeled with Biotin, equal amounts of protein were precipitated using streptavidin agarose beads. The above Western blot was probed with anti-IKKβ antibody to demonstrate the effect of the inhibitor on the nitrosylation of protein IKKβ.

III. Effect of Inhibitor C3 on IKKβ Activity

The inhibition of GSNOR does affect the nitrosylation of IKKβ in A549 cells, but will this nitrosylation affect the activity of IKKβ? To determine if and how nitrosylation of IKKβ would affect its activity and signaling in the NF-κB pathway, cells were treated with C3 and then TNFα. TNFα stimulates the TNF receptor in the plasma membrane which initiates a cascade of kinase activity in the cytoplasm that phosphorylates IKKβ which phopsphorylates the Inhibitor kappa B protein (IκB). This phosphorylation of IκB, (pIκB) activates the ubiquination and proteasome degradation of IκB releasing and therefore allowing the translocation of NF-κB to the nucleus, Figure 5. If the activity of IKKβ is affected, a difference in the extent of phosphorylation of IκB should be detectable. The Western blot in Figure 14 demonstrates the effect of C3 on the phosphorylation of IκB in A549

cells. The amount of phosphorylation of IκB from TNFα stimulated cells appears to be the same as the untreated cells (Figure 14A). But after probing for IκB which identifies both IκB and pIκB, the blot shows a significant increase in total IκB with increasing concentrations of GSNOR inhibitor (n=6 * $p < 0.05$, ** $p < 0.01$) (Figure 14B). The higher amount of total IκB could be due to decreased IKKβ activity or decreased proteasomal degradation of IκBα preventing the release of NF-κB.

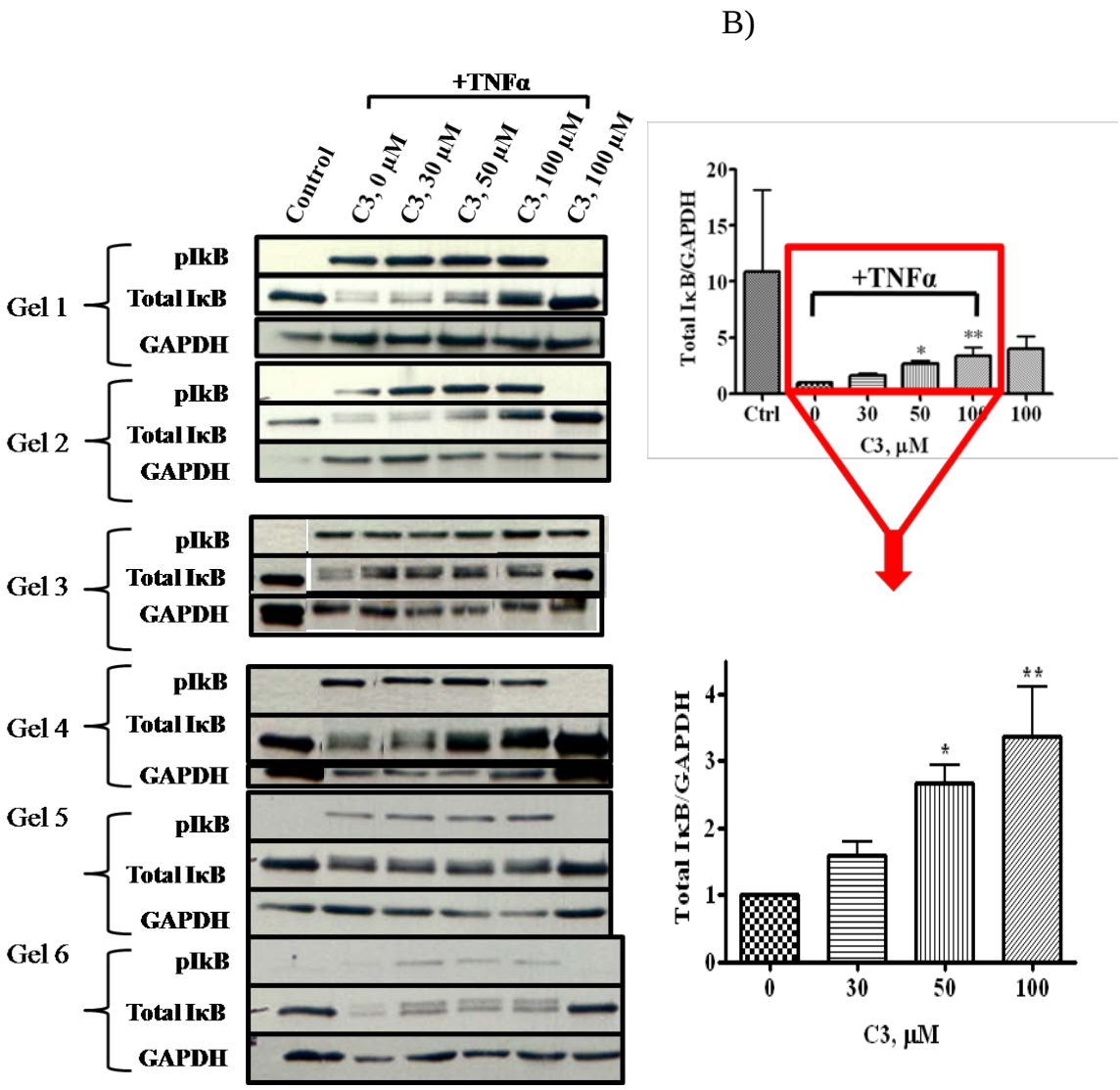
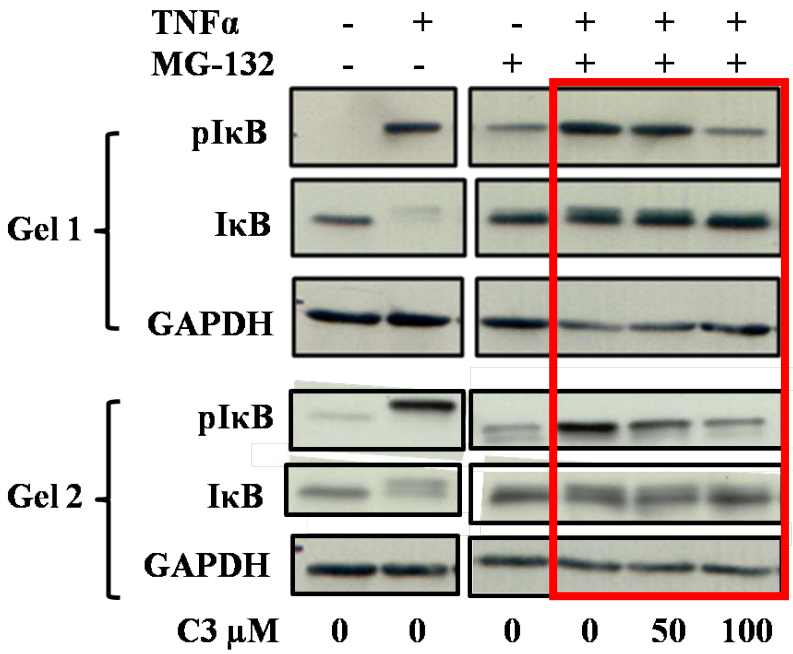


Figure 14. Effect of C3 on the Phosphorylation of IκB. A) Western Blot of A549 cells treated with C3 (0, 30, 50, 100 μM) for 4 hours, then 10 ng/ml of TNFα for 5 minutes or just compound alone or control. B) Analysis of Western Blot using a densitometer (Quantity One Bio-Rad System) and a magnification of the TNFα treated samples, *p< 0.05, **p<0.01 using GraphPad Prism 4 one way anova and Tukey t-test statistics on 6 sets of data from 3 independent experiments.

The addition of proteasome inhibitor, MG-132, in the last hour of the C3 pretreatment (Figure 15) illustrates a much better account of the reaction of the cells to compound C3. The accumulation of pIκB in cells treated with TNFα and MG-132 demonstrates inhibition of proteasomal degradation of IκB. When cells are treated with compound C3, MG-132, and TNFα, the amount of pIκB decreases significantly with increasing concentrations of compound C3 and total IκB appears unchanged. In conclusion, the decreased phosphorylation of IκBα in the presence of compound is most likely due to decreased IKKβ activity and not due to decreased proteasomal activity.



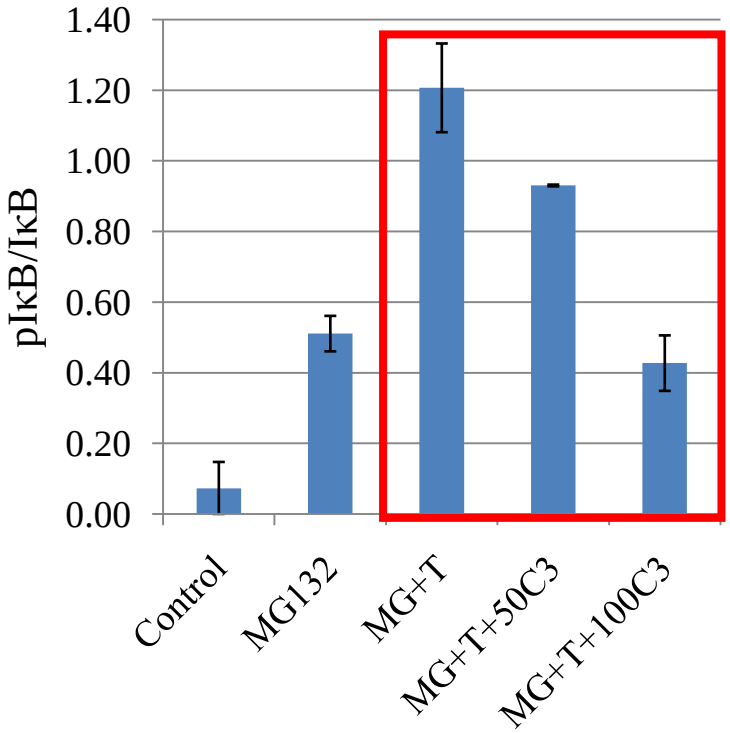


Figure 15. Effect of C3 on the Phosphorylation of IκB in the Presence of a Proteasome Inhibitor. A549 cells were treated with C3 as in Figure 14A plus a known proteasome inhibitor MG-132 1 hour prior to TNFα treatment. Graph average of 2 sets of data.

A dose-dependent increase in nitrosylation of IKKβ was shown in Figure 13. Next, we investigated whether increased nitrosylation affects phosphorylation of IKKβ or total amount of IKKβ. As shown in Figure 16 no change in phosphorylation of IKKβ and no major difference in the accumulation of IKKβ with increasing amounts of C3 was observed. This would suggest that nitrosylation of IKKβ affects its activity but not due to a decrease in phosphorylation.

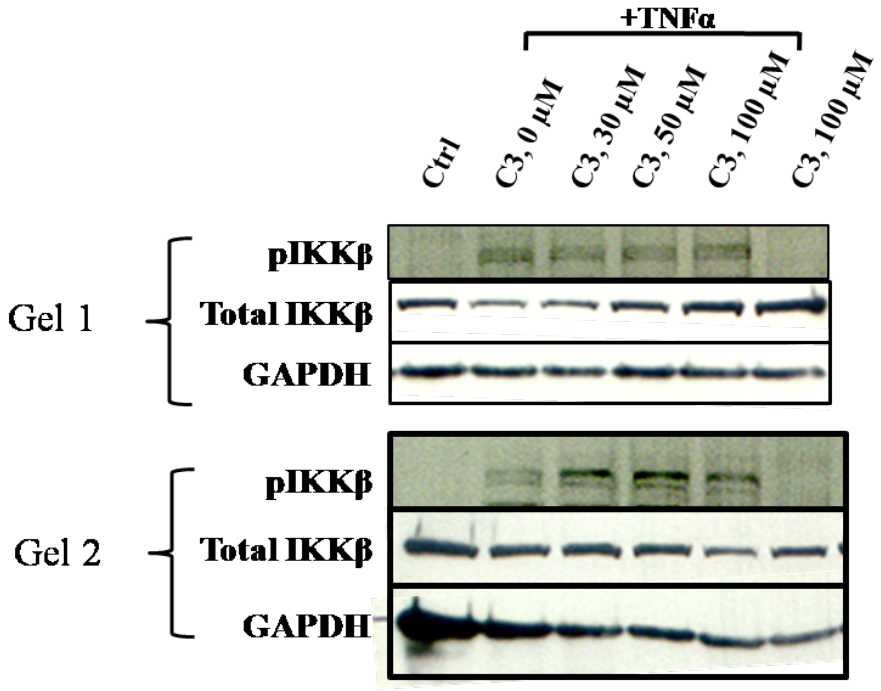


Figure 16. IKKβ Phosphorylation in A549cells. A549 cells treated with 0, 30, 50, 100 μM of C3 for 4 hours and 10 ng/ml of TNFα for 5 minutes assayed by Western blot and probed with anti-pIKKβ, anti-IKKβ and anti-GAPDH antibodies.

IV. Proteins Affected by Inhibited NF-κB Pathway

ICAM is one of the proteins whose expression is regulated by the transcription factor NF-κB. To observe the effects of this protein after inhibiting GSNOR, A549 cells were incubated with inhibitor C3 at varying concentrations and the NF-κB pathway was induced by TNFα. The amount of ICAM-1 produced decreased, with increasing concentrations of inhibitor C3 (Figure 17). This data reveals that C3 inhibits the NF-κB pathway.

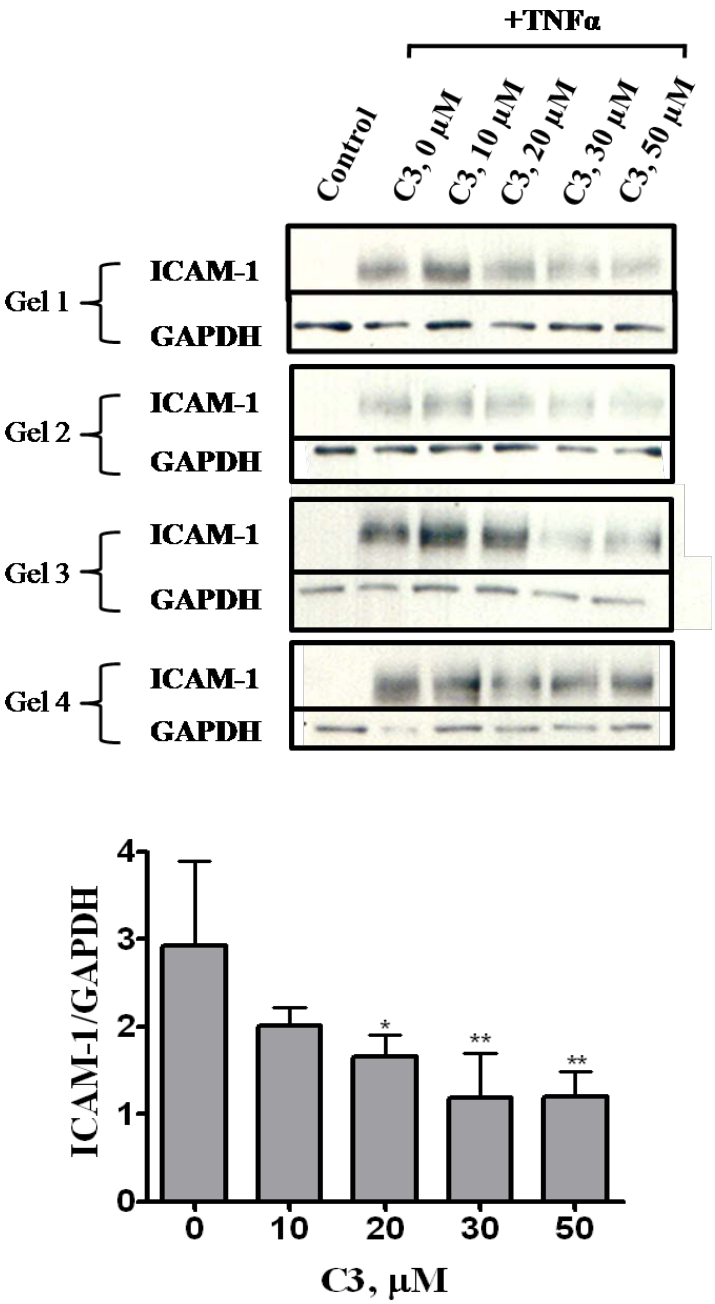


Figure 17. Expression of ICAM-1 is Regulated by NF-κB. Increasing the concentration of inhibitor C3, decreases the amount of ICAM-1 in the A549 cells pre-treated for 6 hours with C3and then an additional 4 hours with TNFα. Statistics on 3 sets of data, *$p<0.05$ and **$p<0.01$.

DISCUSSION

I. Inhibitors of GSNOR

The cytotoxicity of GSNOR inhibition is not known. After 48 hours of exposure, GSNOR inhibitors C2 and C3 demonstrate a moderate cytotoxicity level on the lung epithelial carcinoma cells at low concentrations by hindering cell proliferation. Compounds inhibiting GSNOR activity could be a potential cytotoxic drug at higher concentrations. Cell viability under experimental conditions revealed live cells for the duration of the experiments. These specific inhibitors, C2 and C3 present the possibility of being used for other purposes, since the nitrosylation effects returned to normal levels within 24 hours (Figure 12). Recovery from drug treatment is important to cell viability and will allow flexibility in the formulation of a final marketable drug treatment.

Protein nitrosylation is a post translational modification. Nitric oxide synthases (NOS) generate nitric oxide, which in turn nitrosylates proteins, peptides and amino acids. The S-nitrosylated proteins are called protein-SNO. These protein-SNO and the direct effects of NO regulate several biological functions such as smooth muscle tone, inflammation, apoptosis and others (Figure 18). The nitroso group from the proteins is transferred to glutathione (GSH) forming S-nitrosoglutathione (GSNO). GSNO can then be converted to GSSG and NH_2OH

in the presence of NADH and deplete the GSNO pool. By degradation of GSNO, GSNOR plays an important role in balancing the amount of GSNO and ultimately protein-SNO.

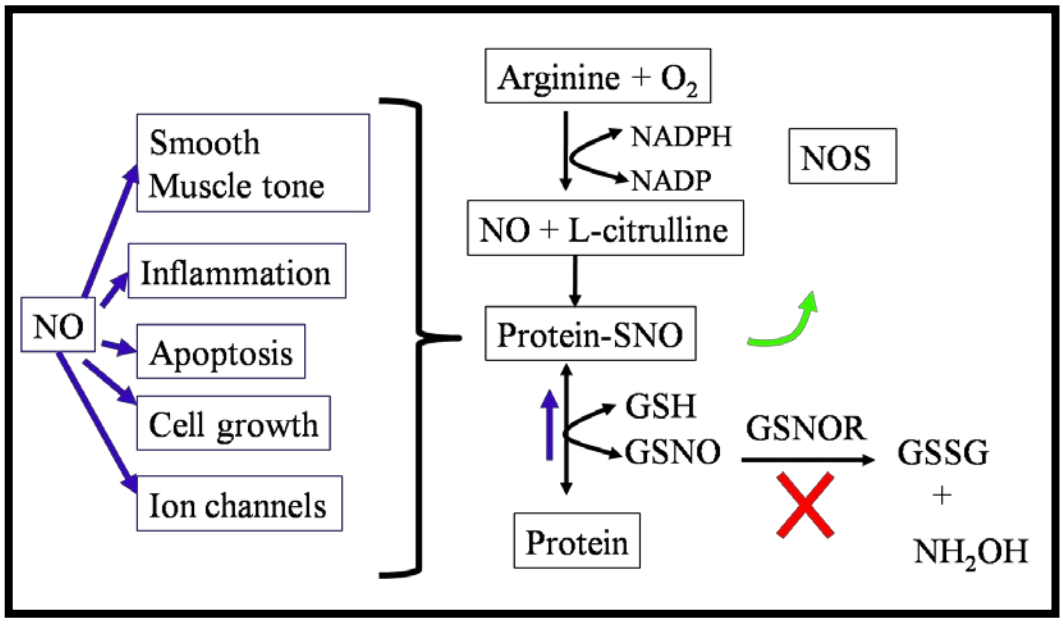


Figure 18. NO Bioactivity and Signaling Pathway. Role of GSNOR inhibition on the NO Signaling Pathway and possibly the bioactivity of NO.

The inhibition of GSNOR will therefore increase the cellular concentration of GSNO and therefore indirectly increases the amount of nitrosylated proteins.

Several GSNOR inhibitors were identified in our laboratory and the aim of this study was to understand their cellular effects. One of the experiments studied the effect of the compound on protein-SNO. As shown in Figure 12, we observed a time dependent increase in protein-SNO. In the same experiment, the treatment with GSNOR inhibitor plus L-NAME inhibited accumulation of nitrosylated

proteins in comparison with the same condition without L-NAME. Since L-NAME is known to inhibit nitric oxide synthases (NOS), this result substantiates that NOS activity is necessary for the observed increase in cellular nitrosylation. This would also indicate that inhibiting GSNOR does not directly increase nitrosylation of proteins but prevents the transfer of NO by decreasing the levels of GSNO.

Next we focused on identification of nitrosylated proteins using protein specific antibodies. As shown in Figure 13 we identified nitrosylation of IKKβ (Figure 19 Step A). So the question raised was whether nitrosylation of IKKβ affects its activity. IKKβ is responsible for phosphorylation of IκBα and phosphorylation of IκBα results in its degradation and activation of NF-κB pathway. Therefore, we studied the phosphorylation of IκBα in the presence of inhibitor C3. After treatment with C3, we expected pIκB levels to be decreased due to nitrosylation of IKKβ. Instead we discovered pIκB amounts were equivalent in C3 treated and untreated cells. However, we did see an increase in total IκBα (Figure 14). To confirm that the accumulation of IκB was due to decreased phosphorylation and not due to inhibition of proteasomal degradation, cells were treated with inhibitor C3 and MG-132, a known proteasome inhibitor. If the decreased degradation of IκBα was due to inhibition of proteasome then the concentration of C3 should not affect the pIκBα in the presence of MG-132. As shown in Figure 15 we observed a dose-dependent decrease in pIκB. Because of this dose-dependent decrease of pIκB, the direct inhibition of proteasomal

degradation by the compounds is eliminated (Figure 19 Step B). Therefore, inhibition of IKKβ activity is most likely responsible for the decreased phosphorylation of IκBα (Figure 19 Step C). So the next question was whether the phosphorylation of IKKβ was affected by nitrosylation (Figure 19 Step D). As seen in Figure 16 we did not detect any change in pIKKβ with different concentrations of C3 (Figure 1).

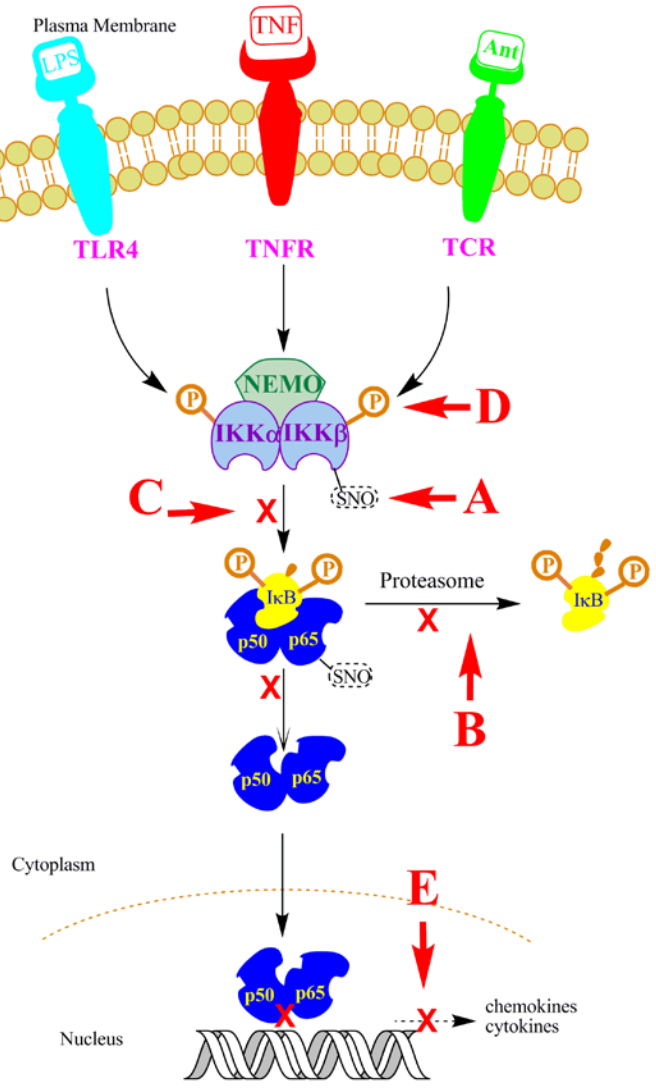


Figure 19. NF-κB Pathway. Effects of inhibiting GSNOR on the NF-κB pathway.

In agreement with our results, inhibition of IKKβ through S-nitrosylation has been documented (Reynaert *et al*., 2004). The activity of IKKβ is inhibited by S-nitrosylation, of the Cys-179 which is between the two phosphorylated serine residues, Ser-177 and Ser-181 (Reynaert *et al*., 2004). The S-nitrosylation of this cysteine could be hindering the active site of the IKKβ or affectin its structural conformation.

Another potential question would be if IKKβ activity is inhibited by direct binding of compound to IKKβ. In a separate experiment done in our lab (data not shown) we showed by an *in vitro* assay that IKKβ activity could not be inhibited by the compounds.

To determine if the decreased degradation of IκBα caused by C3 translated into decreased NF-κB activity we studied the expression of ICAM, one of the proteins whose expression is regulated by the transcription factor NF-κB. We found a dose-dependent decrease in amounts of ICAM-1 with increasing C3 concentration after 10 hours of exposure (Figure 17). In a separate experiment we also studied the effect of C3 on a stable transfected cell line containing a luciferase gene under control of NF-κB, once again we found a dose-dependent decrease in luciferase activity with increasing concentration of C3 (data not shown). Together this data supports the premise that the activity of transcription factor NF-κB is suppressed by inhibiting GSNOR with compound C3 (Figure 19 Step E).

II. Biotechnology

During the experimental procedures, several biotechniques were utilized, Western blots, cell culture, ELISA, and gel filtration. Western blots were a major module for detecting changes in protein expression as a result of treatment with GSNOR inhibitors. Choice of antibodies is critical in Western blots. Antibodies can vary from company to company, and lot to lot. We found that NF-κB pathway proteins antibodies from Cell Signaling Technology were more reliable than other sources. Western blots can be very selective, *ie.* monoclonal antibodies, for the detection of proteins as well as extremely sensitive to the amount of the protein. Monoclonal antibodies are specific for the chosen protein using a unique peptide segment. The monoclonal antibody will only bind to a selected peptide and therefore only one antibody binds to one molecule of protein. This will weaken the signal of the antibody and possibly miss a low abundance protein. Polyclonal antibodies can bind to several different peptides on a protein and therefore provide a larger signal. Polyclonal antibodies might bind other nonspecific proteins. The dilution of the antibody was also critical. If the antibody concentration was too low, the signal would be very low and difficult to obtain. If the concentration of the antibody was too high, the blots would show a lot of nonspecific signals which could interfere with the specific protein you were trying to detect. The protein transfer electrophoresis was also very critical in Western blots. The allotted time for transferring was important. If the transfer was not complete, proteins were left behind in the gel. If the transfer was too long the protein will go through the

membrane and onto the filters. Western blots have more specificity in selecting multiple targets than an ELISA or a UV/Vis spectrophotometry protein assay.

Cytotoxicity testing with both Promega One Assay and the BrdU Assay was important. Both assays are based on different principles.

The data analysis for quantitation using ELISAs are less variable. The issue with ELISAs were that they are time consuming and require developing the conditions for the experiment, selection of antibody, its concentration, standard curve, etc.

Protein Assays can be critical to the analysis of data. Several techniques, such as Western blots, are dependent on the amount of protein loaded onto the gel. Some buffers and detergents used to prepare samples can interfere with the protein assay, *ie*. Bradford protein assay is much more sensitive to the amount of Sodium dodecyl sulfate (SDS) in the sample being analyzed compared to BCA assay where SDS does not interfere with the results.

Cell culture techniques played a major role in obtaining data for examining various hypotheses. Sterility in cell culture is critical since contamination such as bacteria, mycoplasma and other cell lines, invalidate the results. Cell culture is used as a middle step between *in* vitro and whole animal studies. Cell culture will demonstrate the potential results under endogenous conditions compared to *in vitro* results. Cell culture is used for testing several different conditions quickly. Cell culture is much cheaper and humane than whole animal studies. Counting and plating cells is critical for consistency within an

experiment as well as reproducibility between experiments. In cell culture, specific functioning cells can be tested *ie.* hepatocytes of the liver to epithelial cells from the lungs. In our studies two cells lines were chosen, mouse Abelson murine leukemia virus transformed macrophage cells, RAW 264.7 and human lung carcinoma epithelial cell line, A549. The RAW 264.7 cells are known as the cytokine-treated murine macrophage which has been established as a prototype for studying NO-related metabolic pathways for nitrosative stress in mammalian cells (Eu *et al*., 2000).

CONCLUSION

Activation of NF-κB pathway by TNFα is a well defined: IKKβ activation by phosphorylation which then phosphorylates IκB, ubiquination and consequently degradation of pIκB from the cytosolic IκB-NF-κB complex by Proteasome 26. This degradation of IκB exposes the nuclear translocation domain allowing NF-κB to enter the nucleus. Once in the nucleus, NF-κB is the transcription factor and main regulator of the immune response proteins, cytokines and chemokines (Janssen-Heininger *et al*., 2009).

The research provided above supports the suggested hypothesis that inhibiting GSNOR by C3 will suppress the activation of the transcription factor NF-κB indirectly through the regulation of S-nitrosylation of IKKβ.

Future Therapy for Asthma Patients

GSNOR activity is specifically inhibited by C2 and C3. Given that GSNOR has been declared an "important regulator of Human Asthma" (Que *et al*., 2009) managing the activity of this enzyme through inhibition could be a major advancement in Human Asthma relief and other lung diseases (Staab *et al*., 2009) Perhaps with key formulation design of the inhibitor, GSNOR can be regulated using C2 or C3 as a future treatment for asthma.

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Zhang,Y., Keszler,A., Broniowska,K.A., and Hogg,N. (2005). Characterization and application of the biotin-switch assay for the identification of S-nitrosated proteins. Free Radic. Biol. Med. *38*, 874-881.

CURRICULUM VITAE

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EDUCATION

Masters of Science in Biochemistry and Molecular Biology, 2009, Indiana University

Graduate Certificate of Biotechnology, 2006, Indiana University Department of Biochemistry and Molecular Biology

Bachelor of Science in Biochemistry, 1983, Purdue University

SHORT COURSES

AMA: Management Skills and Techniques for New First-Line Supervisors

ACS Short Course: Fundamentals of Experimental Design

The Center for Professional Advancement: Drug Product Stability and Shelf-Life

PUBLICATIONS

Sanghani,P.C., Davis,W.I., Fears,S.L., Green,S.L., Zhai,L., Tang,Y., Martin,E., Bryan,N.S., and Sanghani,S.P. (2009). *Kinetic and cellular characterization of novel inhibitors of S-nitrosoglutathione reductase. J. Biol. Chem.*

Mello T, Nakatsuka A, Fears S, Davis W, Tsukamoto H, Bosron WF, Sanghani SP (2008) *Expression of carboxylesterase and lipase genes in rat liver cell-types.Biochem Biophys Res Commun. 374*, 460-4.

EXPERIENCE

2005 – Current

Research Associate, Indiana University School of Medicine, Department of Biochemistry and Molecular Biology

Masters Research: *Effect of Inhibition of S-nitrosoglutathione Reductase on the NF-κB Pathway*. Investigated the effects of small molecules on the NF-κB signaling pathway, for potential cancer or anti-inflammatory drug; Working with two cells lines, RAW 264.7 murine macrophage cells and A549 lung epithelial cells, studying cell proliferation, apoptosis, nitrosylation of proteins, and effects on inflammatory signaling pathways

Research: Kinetics on a cancer drug with enzymes, developed HPLC and Solid Phase Extraction method to quantitate substrate and product; Kinetic Assay of Carboxylesterase with Benazepril using LC/MS; Isolated hepatic stellate cells from rat liver and tested lysates for different lipase enzymes by Western blot, and activity assays (methods used: UV/Vis Spectrophotometry, Fluorescence Spectrophotometry and HPLC)

Experience in the following Techniques: Protein Expression & Purification in E.coli, Electrophoresis, Western blot, maintenance of several cell lines, Cytotoxicity, ELISA, Immunoprecipitation, Immunostaining, Fluorescence Microscopy, DNA and RNA isolation, Plasmid Purification, Transfections, PCR, and quantitative PCR, HPLC, Solid Phase Extraction, Kinetics, Maldi Mass Spectroscopy, LC/MS

Manage lab: Purchasing equipment and consumables, maintenance and scheduling of equipment; Write and test Protocols, and prepare all materials for the graduate lab classes, Proteomics and Cell Biology, and then assist students during the laboratory class

1983 - 1992

Pitman-Moore Research and Development Pharmaceutical Division
1989 Associate Research Scientist

Directed all NADA stability studies; Designed experiments to obtain data for FDA Submissions; Received and analyzed stability data following GLP; Communicated stability experiment results to product team; Authored NADA stability sections

Discovered a stability problem, to much water in a protein formulation, and helped reformulate a more stable product

Discovered a major storage problem in the diluent of a key product; Developed new product formulations; Supervised full and part time lab technicians

1987 Assistant Research Scientist

Monitored and coordinated NADA stability studies; Maintained database for stability studies; Preformulated and formulated new products; Supervised full-time lab technician

1983 Research Technician

Developed and evaluated protein formulations

Tested formulations using Protein Analysis of protein concentrations by Bio Rad Protein Assay, UV Spectrum, and SDS-PAGE Gels; Lyophilization of protein formulations

Liaison between Formulation Department and Animal Scientists during *in vivo* testing of delivery systems, participated in start up of animal studies and recovery of implants

Maintained animal testing database; Participated in new product evaluation