A Dissertation
entitled
Development of a Reactive Oxygen Species-Sensitive Nitric Oxide Synthase Inhibitor for
the Treatment of Ischemic Stroke
by
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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the
Doctor of Philosophy Degree in
Experimental Therapeutics

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The University of Toledo
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Ischemic stroke is caused by a blockage of the blood flow to the brain resulting in neuronal and glial hypoxia leading to inflammatory and free radical-mediated cell death. Reactive oxygen species (ROS) formed in excess under hypoxic conditions cause protein, DNA and lipid oxidation. Nitric oxide (NO) formed by NO synthase (NOS) is known to be protective in ischemic stroke, however NOS has been shown to ‘uncouple’ under oxidative conditions to instead produce superoxide. Nitrones are antioxidant molecules that are shown to trap ROS to then decompose and release NO. In this study, the PBN-type nitrone **5** was designed such that its decomposition product is a NOS inhibitor, effectively leading to NOS inhibition specifically at the site of ROS production. The ability of **5** to spin-trap radicals and decompose into the putative NOS inhibitor was observed using EPR and LC-MS/MS. The pro-drug concept was tested *in vitro* by measuring cell viability and inhibitor formation in SH-SY5Y cells subjected to oxygen glucose deprivation (OGD). **5** was found to be more efficacious and more potent than PBN, and was able to increase pAkt while reducing nitrotyrosine and cleaved caspase-3 levels. Doppler flowmetry on anesthetized mice showed an increased cerebral blood flow

upon intravenous administration of 1 mg/kg **5**, but a return to baseline upon administration of 10 mg/kg, likely due to its dual nature of antioxidant/NO-donor and NOS-inhibition properties. Mice treated with **5** after permanent middle cerebral artery occlusion (pMCAO) performed better in neurobehavioral assessments and exhibited a > 30% reduction in infarct volume. This efficacy is proposed to be due to higher formation of the NOS inhibitor decomposition product in ischemic tissue observed by LC-MS/MS, resulting in region specific effects limited to the infarct area.

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List of Abbreviations

ΔG_{rxn}	Change in Reaction Gibbs Free Energy
3-NT	3-Nitrotyrosine
5-HT	Serotonin
ACN	Acetonitrile
ADMA	Asymmetric Dimethyl Arginine
ADP.....	Adenine Dinucleotide Phosphate
Akt.....	Protein Kinase B
ANOVA	Analysis of Variance
AUC	Area under the Curve
B3LYP	Becke, 3-parameter, Lee-Yang-Parr
Bax	Bcl-2-Associated X Protein
Bcl-2.....	B-cell Lymphoma-2
BnA	Benzaldehyde
boc.....	<i>Tert</i> -butyloxycarbonyl
br	Broad NMR Peak
BSA.....	Bovine Serum Albumin
CBF	Cerebral Blood Flow
cGMP	Cyclic Guanosine Monophosphate
CD8+	Cluster of Differentiation 8 Positive
$\cdot\text{CH}_3$	Methyl Radical
cm.....	Centimeter
CNS.....	Central Nervous System
COPD.....	Chronic Obstructive Pulmonary Disorder
DFT	Density Functional Theory
dH ₂ O	De-ionized water
DMDO	Dimethyldioxirane
DMEM/F12.....	Dulbecco's Modified Eagle Medium / Ham's F12
DMF.....	Dimethyl Formamide
DMPO.....	5,5-dimethyl-1-pyrroline- <i>N</i> -oxide
DMSO.....	Dimethylsulfoxide
DNA.....	Deoxyribonucleic Acid

DTPA	Diethylenetriaminepentaacetic Acid
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
eNOS	Endothelial NOS
EPR/ESR	Electron Paramagnetic Resonance
eq	Equivalents
ERK1/2	Extracellular Regulated Kinases 1/2
ETC	Electron Transport Chain
EtOAc	Ethyl Acetate
EtOH	Ethanol
FA	Formic Acid
G	Gram
GABA	γ -Aminobutyric Acid
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GHz	Gigahertz
Glu	Glutamate
GSK-3 β	Glycogen Synthase Kinase 3 Beta
H-Bond	Hydrogen Bond
HBSS	Hank's Buffered Saline Solution
HEPES	Hydroxyethyl Piperazineethanesulfonic Acid
hfsc	Hyperfine Splitting Constant
HO ₂ ·	Hydroperoxyl Radical
HO-1	Heme Oxygenase-1
HPLC-PDA	High Pressure Liquid Chromatography – Photodiode Array
hr	Hour
HRMS	High-Resolution Mass Spectrometry
HRP	Horseradish Peroxidase
Hz	Hertz
IBA	4(1 <i>H</i> -Imidazol-1-yl)benzaldehyde
IL-1 β	Interleukin-1 Beta
iNOS	Inducible NOS
<i>J</i>	J-Coupling Constant
kcal/mol	Kilocalorie per Mole
kDa	Kilodalton
kg	Kilogram
K _i	Dissociation Constant
L-NAME	<i>N</i> ω -nitro-L-arginine Methyl Ester
L-NMMA	L- <i>N</i> (γ)-monomethylarginine

LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
LPS.....	Lipopolysaccharide
m	Multiplet NMR Peak
M.....	Parent Mass / Molar
m-CPBA.....	<i>m</i> -Chloroperbenzoic Acid
<i>m/z</i>	Mass-to-charge Ratio
MCA	Middle Cerebral Artery
MCAO.....	MCA Occlusion
MeOH	Methanol
MHz	Megahertz
MI.....	Myocardial Infarction
min	Minute
mg	Milligram
μL.....	Microliter
mL.....	Milliliter
mM.....	Millimolar
MNP	2-methyl-2-nitrosopropane
MRM.....	Multiple Reaction Monitoring
msec	Millisecond
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
mW.....	Milliwatt
N.....	Normal
NADPH.....	Nicotinamide Adenine Dinucleotide Phosphate
NDS.....	Neurological Deficit Scoring
NIH	National Institute of Health
nm	Nanometer
NMR	Nuclear Magnetic Resonance Spectroscopy
nNOS.....	Neuronal NOS
NO.....	Nitric Oxide
NO ₂	Nitrogen Dioxide
NOS.....	Nitric Oxide Synthase
NPA.....	Natural Population Analysis
NXY-059.....	Disufenton Sodium
O ₂ ^{•-}	Superoxide
•OH.....	Hydroxyl Radical
ONOO ⁻	Peroxynitrite Anion
ONOOH	Peroxynitrous Acid
OGD.....	Oxygen Glucose Deprivation
•OX.....	Oxygen-centered Radical
p-	Phospho
PBN.....	<i>N-tert</i> -butyl- <i>α</i> -phenylnitrone
PBS	Phosphate Buffered Saline

PCM	Polarizable Continuum Model
PDB	Protein Data Bank
PEG	Polyethylene Glycol
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-Kinase
PIP ₃	Phosphatidylinositol (3,4,5)-trisphosphate
PGs	Prostaglandins
pKa	Acid Dissociation Constant
pMCAO	Permanent MCAO
ppm	Chemical Shift
RIPA	Radioimmunoprecipitation Assay
ROS	Reactive Oxygen Species
rt	Room Temperature
RT	Retention Time
s	Singlet Peak
S-PBN	Sulfonyl-PBN
S2	Spin Contamination
SAINT-I/II	Stroke–Acute Ischemic NXY Treatment I/II
sec	Second
SEM	Standard Error of the Mean
Ser	Serine
SDMA	Symmetric Dimethyl Arginine
SDS	Sodium Dodecyl Sulfate
S _N 2	Bimolecular Nucleophilic Substitution
SOD	Superoxide Dismutase
STAZN	Stilbazulenyl Nitrone
TBN	Tetramethylpyrazine Nitrone
TBST	Tris Buffered Saline, 0.1% Tween-20
<i>tert</i>	Tertiary
TFA	Trifluoroacetic Acid
TLC	Thin Layer Chromatography
TLR4	Toll-like Receptor 4
TNF- α	Tumor Necrosis Factor-Alpha
TPA	Tissue Plasminogen Activator
Tris	Tris(hydroxymethyl)aminomethane
Trp	Tryptophan
TTC	2,3,5-Triphenyl-tetrazolium Chloride
XO	Xanthine Oxidase
ZPE	Zero-point Vibrational Energy

List of Symbols

$\sim$		Approximately
$\cdot^-$		Radical Anion
$\pm$		Plus-Minus
$^{\circ}\text{C}$		Degrees Celsius
Δ		Change in
δ		Chemical Shift
K		Degrees Kelvin
λ		Wavelength
π		Pi-molecular Orbital
μ		Micro
$\text{\AA}$		Angstrom

Chapter 1

Design and Synthesis of an ROS-Sensitive NOS Inhibitor

Introduction

Stroke remains one of the leading causes of death and disability in the United States, most notably ischemic stroke which accounts for over 80% of all stroke cases. Despite decades of promising research, clinical trials of potential therapeutics have not successfully led to novel treatments for the devastating effects of cerebral ischemia.¹⁻³ Ischemic stroke is associated with inflammatory and free radical-mediated cell death due to obstruction of blood flow to the brain. Reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot -}$) are generated under hypoxic and inflammatory pathologies by the mitochondrial electron transport chain (ETC), NADPH oxidase, and xanthine oxidase (XO). This oxidative burst causes direct DNA, protein, and cell membrane damage which ultimately leads to cell death in the core and penumbra of the ischemic lesion. Additionally, $O_2^{\cdot -}$ is known to cause vasoconstriction independent of its effect on nitric oxide (NO),⁴ which is detrimental to patient outcome. Increased levels of oxidative stress markers and decreased levels of antioxidant markers have a strong correlation with worse ischemic stroke outcome.⁵

NO is a free-radical, paracellular signaling molecule produced by Ca^{2+} -dependent endothelial NO synthase (eNOS) and neuronal NOS (nNOS), and inducible NOS (iNOS).

Canonical NO signaling involves the increase in cGMP through activation of guanylyl cyclase to cause vasodilation through vascular smooth muscle relaxation. It has been recently discovered that NO is also involved in GABA⁶ and 5-HT neurotransmission,⁷ neurogenesis,⁸ mitochondrial signaling,⁹ and post-translational protein modification.¹⁰ During cerebral ischemia a dramatic increase in intracellular Ca²⁺ causes increased NOS activity through calmodulin binding concomitant with the generation of ROS and activation of pro-apoptotic pathways. The overall role of NO in ischemic stroke is still unclear; inhaled NO was shown to be clinically ineffective,¹¹ while endogenous NO generated by NOS is observed to have beneficial effects.¹² NOS can uncouple under ischemic conditions through direct oxidation or oxidation of cofactors to instead produce O₂^{•-}, which is scavenged by NO to form the highly reactive peroxynitrite (ONOO⁻). S-glutathionylation of eNOS, a redox-dependent post-translational modification, was shown to reversibly regulate its uncoupling based on its local redox environment.¹³ This phenomenon was initially observed with eNOS,¹⁴ but has since been detected from each NOS isoform.^{15,16}

Importantly, Guzik et al. were able to show the pan-NOS inhibitor N^ω-nitro-L-arginine methyl ester (L-NAME) subdued O₂^{•-} production in tissues with NOS dysfunction.¹⁷ L-NAME administered to tissue with functional NOS increased O₂^{•-} production however, which suggests a targeted approach to dysfunctional NOS inhibition would be preferable to total NOS inhibition. Isoform specific inhibitors for nNOS or iNOS have shown reduced *in vivo* infarct volume in animal models, while pan-NOS inhibitors exhibit mixed results.¹⁸ This may be due to the inhibition of functional NOS as well as uncoupled NOS which can cause global adverse effects that limit their protective

effects in ischemia. Even so, it was found that NOS inhibition, specifically that of nNOS, improves the utilization of O₂ during ischemia by improving the average O₂ supply/consumption ratio in brain tissue exposed to ischemia/reperfusion, which was associated with a reduction in infarct size.¹⁹ The formation of NO by NOS – or O₂^{•-} by uncoupled NOS – requires O₂, thus under ischemic conditions it may be beneficial for the scarce O₂ to be instead utilized by other processes. NOS inhibition has shown pre-clinical efficacy in improving pathology in other neurological conditions such as epilepsy,^{20,21} Parkinson's disease²² and spinal cord injury.²³ Not surprisingly, each of these pathologies have also been linked to increased oxidative stress, calling into question the full role of the NOS isozymes in these pathologies.

Antioxidant molecules that scavenge oxidative species such as Edaravone,²⁴ Ebselen,²⁵ and uric acid²⁶ have shown pre-clinical promise in ischemia, but have fallen short in clinical applications. Nitrones (**Figure 1-1**) are synthetic antioxidants that are shown to both scavenge ROS and produce NO upon decomposition²⁷ to yield neuroprotective effects under ischemic conditions. The nitrone NXY-059 (Cerovive[®]) had advanced to phase III clinical trials, showing good safety and tolerability before it was deemed ineffective in the treatment of acute ischemic stroke.²⁸ No adverse effects could be found for NXY-059, also called OKN-007 and HPN-07, in patients with ischemic stroke, even at high doses.²⁹ Incidence of adverse effects were similar for drug and placebo groups, which included fever, headache, and hypertension. Linear, *N-tert-butyl- α -phenylnitron*e (PBN)-type nitrones have been extensively developed for use in pre-clinical models of stroke including innovative derivations such as a tetramethylpyrazine nitrone (TBN), which possesses anti-platelet activity,³⁰ and a PBN-

containing, PEGylated nanoparticle capable of extended exposure.³¹ In addition to ischemia/reperfusion injury, nitrones have been explored as therapeutics in models of Parkinson's disease, Huntington's disease,³² and epilepsy.^{33,34}

Nitrones are conventionally synthesized by oxidation of a secondary amine by *m*-chloroperbenzoic acid (m-CPBA),³⁵ Dimethyldioxirane (DMDO),³⁶ or transition metals such as Mo(IV) or W(IV),³⁷ or by condensation of *N*-monosubstituted hydroxylamines with carbonyl compounds. Hydroxyphenyl PBN-type nitrones were recently synthesized via a one-step microwave reaction from their corresponding benzaldehydes and *tert*-butyl hydroxylamine in acetic acid.³⁸ Nitrones have seen extensive synthetic modifications since the development of NXY-059, primarily increasing their antioxidant potential while simultaneously increasing hydrophobicity. Examples of this include a Quinolinyl nitrone,³⁹ cholesterol nitrones,⁴⁰ bi-cyclic nitrones,^{41,42} and the highly lipophilic STAZN.³² Each of these molecules was assayed for their neuroprotective properties in various pathologies involving oxidative stress. Recent developments have seen alternative approaches to simply increasing reactivity, such as imparting mitochondria localization,⁴³ anti-parasitic activity,⁴⁴ nanoparticle conjugation,⁴⁵ and intramolecular adduct cyclization⁴⁶ and stabilization.⁴⁷ Additionally, nitrones are being utilized to detect the localization of free radicals beyond traditional ESR spectroscopy; Conversion of the conjugated nitrone STAZN to its aldehyde was used for colorimetric detection and localization of oxidative stress in animals subjected to ischemia/reperfusion.⁴⁸ The cyclic nitrone DMPO forms adducts with DNA and proteins that are stable enough to be detected by a DMPO-specific antibody using a technique called immuno-spin trapping.⁴⁹ DMPO-biomolecule adducts were observed by immunohistochemistry and western

blotting after ischemia/reperfusion to identify the localization of oxidative stress and its targets. These advancements depict the ongoing efforts to improve nitrones as both biological probes and therapeutics for oxidative pathologies.

Synthetic nitrones and NOS inhibitors have thus far failed to yield favorable clinical outcomes in diseases mediated by oxidative pathologies. This study aims to resolve their limitations through a pro-drug approach to the elimination and prevention of oxidative species. The molecule described herein, **5**, is the first nitron to be designed based on its radical adduct decomposition product. This decomposition product is a putative NOS inhibitor that will effectively be formed at the site of oxidative stress, selectively targeting dysfunctional, uncoupled NOS. In this study we use an *in silico* computational approach to design **5** and its product **6** before advancing to *in vitro* and *in vivo* models of ischemic brain pathology.

Figure 1-1. Representative structures of linear and cyclic nitrones, and NOS-inhibitors.

Results

Thermodynamics of Free Radical Addition to **5**

It has been shown that oxygen-centered radical addition to PBN nitrones causes nitronyl bond cleavage to form an NO-donating *tert*-butyl hydroxylamine and a benzaldehyde.⁵⁰ The amidine-containing NOS inhibitor 1400W exhibits strong selectivity for iNOS, while also showing inhibition of nNOS and eNOS at higher concentrations. For this proof-of-concept study, the PBN-type nitron **5** was designed with the goal of forming the putative NOS inhibitor **6**, modeled from 1400W, after reaction with oxygen-centered radicals (**Figure 1-2**). While the mechanism of this decomposition has not been fully elucidated, NO-release has been repeatedly shown from both linear and cyclic nitrones. Amidine moieties typically exhibit an NH pKa > 10, thus the protonated forms of **5** and **6** were used for all *in silico* calculations. Multiple biologically relevant oxygen-centered free radicals were chosen for calculation of reaction thermodynamics at the PCM/B3LYP/6-31+G(d,p)//B3LYP/6-31G(d) level of theory. **Figure 1-3** shows calculated geometries of **5** and adducts formed from O₂^{•-}/HO₂[•] (pKa 4.8), [•]OH, and ONOO[•]/ONOOH (pKa 6.8). Calculated ΔG_{rxn} for radical addition to **5** and PBN are summarized in **Table 1.1**.

Optimized geometries of O₂^{•-} addition to **5** calculated by Gaussian 09 predict proton abstraction of the amidine-NH to protonate the peroxy anion adduct. While this resulted in a lower ΔG_{rxn} (1.6 kcal/mol), it is more likely that the peroxy anion would abstract a proton from solution. Indeed, when the calculation was performed with two explicit water molecules, the ΔG_{rxn} of **5**- O₂^{•-} was closer to that of PBN- O₂^{•-} (12.6 kcal/mol and 19.8 kcal/mol, respectively). Calculation of the deprotonated form of **5**

predicts a less favorable ΔG_{rxn} for $\text{O}_2^{\cdot -}$ addition (19.3 kcal/mol, > 10x that of protonated **5**) which illustrates the predicted role of the amidine moiety in affecting the reactivity of the nitron with anionic radicals.

Peroxynitrite and its conjugate acid ONOOH are highly oxidizing species that have been shown to react with nitrones to yield radical products as detected by EPR.⁵¹ The ΔG_{rxn} of potential triplet products were calculated, and predicts a favorable reaction of **5** with the relatively stable *cis*-ONOO⁻ to give NO₂, as compared to an unfavorable reaction with PBN (-6.7 kcal/mol and 32.5 kcal/mol, respectively). Alternatively, ONOO⁻ could act as a nucleophile, and attack the nitronyl-carbon to form singlet products. Calculations of these reactions predict them to be highly exoergic: ΔG_{rxn} -50.5 kcal/mol for ONOO⁻ addition to **5**, (for PBN: ΔG_{rxn} -38.1 kcal/mol for ONOO⁻ addition). While these calculations predict nucleophilic attack to be the more favorable reaction mechanism with PBN and **5**, they do not agree with previous EPR observations and those herein, as only singlet, and thus EPR-silent products would be formed from such reactions. Overall, the effect of the H-bond donating amidine group is predicted to have favorable effects on the ΔG_{rxn} with anionic ROS compared to PBN. This is in line with previous observations with nitrones containing intramolecular H-bond donors.^{52,53}

Figure 1-2. General scheme for the formation of the putative NOS inhibitor **6** from the nitrone **5** after reaction with an oxygen-centered free radical ($\cdot\text{OX}$) to release NO.

Figure 1-3. Optimized structures including bond lengths, and charge and spin densities (in parenthesis) for **5**, its proposed free radical adducts, and calculated ΔG_{rxn} , formed from $\text{O}_2^{\cdot -}/\text{HO}_2^{\cdot}$, $\cdot\text{OH}$, and HOONO (triplet products) at the PCM/B3LYP/6-31+G(d,p)//B3LYP/6-31G(d) level of theory.

Table 1.1. Calculated free energies of reaction (ΔG_{298K} , kcal/mol) of oxygen-centered free radical species to form nitronyl spin adducts at the PCM/B3LYP/6-31+G(d,p)//B3LYP/6-31G(d) level of theory

<u>Radical Adduct</u>	<u>PBN</u>	<u>5</u>
-O ₂ ⁻	19.8	1.6(12.6) ^a
-O ₂ H	2.1	3.2
-OH	-32.7	-31.1
-OONO ^b	32.5	-6.7
-HOONO ^c	-23.4	-20.4

^aWith two explicit water molecules

^bTriplet products of a O-3 *cis*-OONO adduct

^cTriplet products of a O-3 *trans*-HOONO adduct

Predictive Binding of **5** and **6** with NOS Isozymes

To predict if the nitron **5** and its decomposition product **6** interact with the active site of each NOS isoform, docking using Swissdock.ch was performed. PDB structures of each NOS isoform bound with native ligand L-arginine, and those with bound 1400W were used (6 structures total). Results of predicted interaction with the orthosteric binding site, the active site Glu which coordinates binding ligands over the catalytic heme, are summarized in **Table 1.2**. Both **5** and **6** were predicted to H-bond with the active site Glu, and a nearby Trp to position them over the heme (**Figure 1-4**). The ΔG of ligand binding for **5** and **6** were found to be similar for each NOS isoform; the most favorable interaction with iNOS, and least favorable with nNOS. **5** was found to have slightly higher affinity than **6** for each isozyme, which is undesirable if the inhibitor function of the drug is to be ‘unmasked’ by ROS. Both molecules gave similar predicted ΔG as that of L-arginine for each structure, with the exception of nNOS in which **5** and **6** had more favorable predicted binding for both nNOS structures. 1400W showed the most favorable binding for each structure, although seemingly similar to L-arginine for iNOS binding ΔG in spite of its high affinity ($K_i = 7$ nM). The primary amine of 1400W was observed to H-bond with a carboxyl group of the active-site heme, which may account for its increased affinity as compared to the other ligands. Upon calculating favorable interactions with NOS active sites and promising ΔG_{rxn} for free radical reactions, the compound **5** was advanced for synthesis. These *in silico* methods can be used for future development of ROS-activated NOS inhibitors to screen compounds prior to their synthesis.

Table 1.2. Predicted free energies of binding (ΔG_{298K} , kcal/mol) of ligands with NOS isozymes (L-arginine/1400W x-ray crystallographic structures)^a

<u>Ligand</u>	<u>eNOS</u> ^b	<u>iNOS</u> ^c	<u>nNOS</u> ^d
L-arginine	-10.32/-9.31	-10.64/-10.21	-7.78/-8.43
1400W	-10.37/-10.34	-10.68/-11.56	-8.62/-10.26
5	-9.99/-10.11	-10.07/-10.75	-8.29/-9.88
6	-9.73/-9.88	-9.76/-10.09	-8.83/-9.77

^aDocking performed by Swissdock.ch^{54,55}

^beNOS L-arginine PDB: 4D1O; 1400W PDB: 1FOI

^ciNOS L-arginine PDB: 1NSI; 1400W PDB: 1QW5

^dnNOS L-arginine PDB: 4D1N; 1400W PDB: 1QWC

Figure 1-4. Most energetically favored binding structures of L-arginine (**A**), 1400W (**B**), **5** (**C**) and **6** (**D**) with nNOS (PDB: 1QWC) calculated by Swissdock.ch and visualized by UCSF Chimera.

Synthesis and Characterization of **5**

The four-step synthesis of **5** was carried out with a favorable overall yield of 52.5% (**Figure 1-5**). The intermediates allow for multiple points of derivatization for the quick synthesis of a focused library. The boc-protected benzyl alcohol **1** was purchased for the synthesis herein, however the precursor 3-(aminomethyl)-Benzenemethanol can be purchased at low cost (5 g for \$400) for large-scale synthesis. Activation of the boc-protected benzyl alcohol **1** by Appel bromination allows for substitution with *tert*-butyl amine (**3**), then further de-protection permits substitution with an amidine (**4**). These two steps can be used for future derivatization to modulate activity of the nitron and NOS-inhibitor aspects of the molecule, respectively. The final step is oxidation of the secondary amine to the nitron **5** by NaWO₄-H₂O₂. While no oxidation of the amidine was observed, future intermediates may be sensitive to this oxidation step. Analysis by ¹H and ¹³C NMR, HRMS, and HPLC-PDA showed the successful synthesis of **5** at > 95% purity. The intermediate **4** was found to have very weak absorbance, even at its λ_{max} of 260 nm, but following oxidation to the nitron **5**, the λ_{max} shifted to 289 nm and gave strong absorbance character. The formation of the nitron results in π -electron conjugation from the benzene ring to continue through the nitronyl N-O bond which greatly affects its absorbance spectra. This observation correlates with the breaking of this conjugation and subsequent decrease in absorbance and λ_{max} upon reaction of **5** with free radicals.

Figure 1-5. Synthetic scheme of 5 and intermediates.^a

^a(**a.**) CBr₄, PPh₃, CH₂Cl₂, 0 °C → rt 30 hr; (**b.**) *tert*-butylamine, K₂CO₃, DMF, 3 hr; (**c.**)

(i) TFA, CH₂Cl₂, 0 °C, 2 hr; (ii) ethyl acetimidate, EtOH, rt, 12 hr; (**d.**) H₂O₂,

Na₂WO₄(H₂O)₂, MeOH, 0 °C → rt, 12 hr.

5 EPR Spin Trapping and Formation of 6

EPR characterization of radical adducts of **5** were carried out using conventional ROS-generating systems. Spectra for PBN were obtained using the same systems, and matched previous hyperfine splitting constant (hfsc) observations. Resulting EPR spectra from addition of **5** to $O_2^{\cdot-}/HO_2^{\cdot}$, $^{\cdot}OH/CH_3$, and $ONOO^-/HCl$ systems are shown in **Figure 1-6**. Solutions of $O_2^{\cdot-}$ and $^{\cdot}OH$ generated oxygen and carbon-centered radicals whose spectra closely resemble that of PBN. There was no observable difference in signal intensity between **5** and PBN, which suggests a similar reactivity, and is in agreement with the *in silico* ΔG_{rxn} calculations above. Interestingly, an identical three-line EPR signal was observed for both **5** and PBN in the presence of $ONOO^-/HCl$ (no signal was observed for nitron or $ONOO^-/HCl$ alone). There is no literature precedent for the formation of doublet or triplet products from $ONOO^-$ and PBN-type nitrones. Due to the absence of H_{β} splitting, these spectra are hypothesized to pertain to a *tert*-butyl nitroso formed after nitronyl N-C bond cleavage. These products could result from addition of free-radical species to the nitron, or nucleophilic attack to the nitron followed by decomposition, then oxidation of the *tert*-butyl hydroxylamine to give an EPR-visible species. Further investigation into the mechanism behind the observed spectra is needed. Additionally, the $ONOO^-$ system was only attempted at pH ~ 6. This is more physiologically relevant than the basic pH of $ONOO^-$ alone, however subjecting **5** to EPR with $ONOO^-$ at varying pH may elucidate the involvement of $ONOOH$ versus $ONOO^-$.

Solutions of the **5**-ROS systems used to acquire EPR spectra were replicated, and let incubate at 37 °C for 24 hr before analysis by HPLC-PDA at 254 nm (**Figure 1-7**). The ROS systems were also replicated with PBN, and the formation of benzaldehyde was

similarly observed, agreeing with previous observations.⁵⁰ Pure PBN gave a peak at 6.50 min with λ_{max} at 294 nm, while pure benzaldehyde eluted at 6.67 min with a 244 nm λ_{max} . These differences allowed a clear discrimination between the two molecules, and allowed for the confirmation of PBN decomposition in each radical system that was then used to explore **5** decomposition. A new peak at 3.4 min was observed for each of the three radical systems tested (**5** retention time [RT] = 5.4 min). This more-polar compound was formed in varying amounts for each system, and was found to be the only peak in the **5**-ONOO⁻/HCl system. Additionally, the λ_{max} of the new peak was lower than that of **5** (249 nm vs 289 nm), suggesting a loss of conjugation likely due to nitronyl N=C modification or cleavage, as was observed in the difference between the intermediate **4** and nitrone **5**. Low-resolution LC-MS/MS was performed on the **5**-ONOO⁻/HCl solution, and found a strong signal of an m/z matching the predicted mass of the aldehyde **6** ($M + H = 177$; $M + \text{NH}_4 = 195$; $M + K = 215$). This m/z was used to create an MRM analysis by fragmentation. LC-MS/MS analysis of the other **5**-ROS solutions using this MRM showed the presence of **6** in each. These data suggest the ability of **5** to trap ROS and decompose to the putative NOS inhibitor **6**.

Isolation and Characterization of **6**

The **5**-ONOO⁻/HCl system was mimicked at a larger scale to produce sufficient quantity of **6** for isolation and characterization. Interestingly, 0.50 eq of ONOO⁻ was enough to convert nearly all **5** to **6** as observed by HPLC-PDA (95.4 %) after one hour. pH was observed to be critical to this reaction; it was initially attempted in dH₂O without the addition of HCl for the ease of purification, but this resulted in no observable **6** formation, and the products formed that were not identified. The **5**-ONOO⁻/HCl reaction

was extracted with CH₂Cl₂, and then the aqueous fraction was lyophilized to give a white solid containing **6** and various salts. Addition of ACN to this solid followed by filtration allowed the insoluble salts to be removed, and **6** isolated. ¹H NMR in CD₃OD showed the absence of the nitronyl-H_β and *tert*-butyl group, and a new peak at 10.0 ppm which is characteristic of a benzaldehyde (**Figure 1-8**). LC-MS/MS analysis of the isolated **6** yielded the same parent mass and fragmentation pattern as was previously found for **5**-ONOO⁻/HCl.

Figure 1-6. X-Band EPR spectra and simulations of PBN or **5** (35 mM) in the presence of $\text{O}_2^{\cdot-}/\text{HO}_2^{\cdot}$ (KO_2 in 20% PBS/DMSO; [A] PBN- $\text{HO}_2^{\cdot}$ adduct, a_{N} : 13.9, $a_{\text{H}\beta}$: 1.91; $a_{\text{H}\gamma}$: 0.42; [B] **5**- $\text{HO}_2^{\cdot}$ adduct, a_{N} : 14.19, $a_{\text{H}\beta}$: 2.48; $a_{\text{H}\gamma}$: 0.52); $\cdot\text{OH}/\text{CH}_3$ ($\text{Fe}^{2+}/\text{H}_2\text{O}_2$ in 30% PBS/DMSO; [C] PBN- $\cdot\text{OH}$ adduct, a_{N} : 15.63, $a_{\text{H}\beta}$: 3.41; $a_{\text{H}\gamma}$: 0.27; $\cdot\text{CH}_3$ adduct, a_{N} : 14.30, $a_{\text{H}\beta}$: 2.58; [D] **5**- $\cdot\text{OH}$ adduct, a_{N} : 15.56, $a_{\text{H}\beta}$: 3.24; $a_{\text{H}\gamma}$: 0.61, [30.6%]; **5**- $\cdot\text{CH}_3$

adduct, a_N : 14.24, $a_{H\beta}$: 2.41, [69.4%]), and $ONOO^-/HCl$ (10 mM, pH~6 in 30% PBS/DMSO;[**E,F**] nitroxyl, a_N : 12.80).

Figure 1-7. HPLC-PDA analysis of free radical reactions with PBN and **5**. (A) HPLC chromatograms of PBN (RT = 6.50 min) and benzaldehyde (BnA; RT = 6.65 min). (B) Chromatograms of PBN incubated with the incubated radical system for 24 hr. Significant BnA formation was observed for KO₂ and ONOO⁻ systems, with minor

formation in the Fe-H₂O₂ system. (C) HPLC chromatograms of **5** (RT = 5.40 min) and **6** (RT = 3.36 min). (D) Chromatograms of **5** incubated with the incubated radical system for 24 hr. Minor **6** formation in the Fe-H₂O₂ system, and significant **6** formation was observed for KO₂ and ONOO⁻ systems with no observed **5** remaining in the ONOO⁻ system.

Figure 1-8. ¹H-NMR (CD₃OD, 400 MHz) of **6** isolated from the **5**-ONOO⁻/HCl system.

The peak at δ 10.04 (1H, s) is characteristic of a benzaldehyde.

Discussion

NOS presents a unique target in the treatment of ischemia due to seemingly conflicting results from NOS inhibition or genetic knockouts, which have shown both beneficial^{18,56-58} and deleterious effects,⁵⁹⁻⁶¹ and may additionally have sex-specific outcomes.⁶²⁻⁶⁴ Indeed, the precise design criteria for potent and selective NOS inhibitors has seen significant exploration to minimize off-target effects.⁶⁵ The co-administration of NOS inhibitors with nitrones for treating neuronal pathology has been previously explored,⁶⁶ as well as administration of a nitron to NOS knockout animals,⁶⁷ each of which show complimentary beneficial effects.

The active constituent of NXY-059 used in the SAINT-I/SAINT-II clinical trials for acute ischemic stroke may not have been the drug itself, but a decomposition product, *N-tert*-butylhydroxylamine,⁶⁸ formed after the oxidation of the nitron, and cleavage of the N-C bond. It has been repeatedly shown that the nitron PBN decomposes to release NO and benzaldehyde under oxidative conditions,⁵⁰ and that this mechanism may account for much of its therapeutic effects.⁶⁹ Bi-functional nitrones are actively being developed to treat ischemic stroke, including a tetramethylpyrazine-functionalized nitron that was shown to reduce ADP-induced platelet aggregation.³⁰ However, until now there has not been an attempt to design nitrones from their decomposition products as pro-drug molecules that are activated under oxidative conditions.

With the goal of forming a putative NOS inhibitor, and knowing the oxidative decomposition product would result in formation of a benzaldehyde, the molecule **6** was designed to structurally mimic the NOS inhibitor 1400W. Amidine-containing NOS inhibitors such as 1400W have been shown to be irreversible NOS inhibitors through a

heme-oxygenase mechanism that does not cause breakdown of the inhibitor.^{70,71} It was then hypothesized that this benzaldehyde would be formed from the nitrone **5**. The structure of **5** and its synthesis are such that future derivatives can be rapidly made to separately modulate ROS reactivity and NOS affinity and selectivity. The screening of future derivatives can begin *in silico* as outlined herein, by predicting reactivity to biologically relevant ROS, and interaction with NOS isozyme active sites. These approaches have been taken separately for the design of nitrones⁵² and NOS inhibitors,⁶⁵ and yielded predictions that agree with subsequent observations.

ROS Reactivity Calculations

Gaussian calculations at the PCM/B3LYP/6-31+G(d,p)//B3LYP/6-31G(d) level of theory of ROS adducts to **5** yielded similar ΔG_{rxn} to that of PBN except for the anionic species $\text{O}_2^{\cdot -}$ and ONOO^- that are capable of H-bonding and proton abstraction. It is still unknown if, at neutral pH, $\text{O}_2^{\cdot -}$ becomes protonated before attack of the nitrone (pKa 4.6), if it reacts with the nitrone and the subsequent peroxy adduct would become protonated, or if the nitrone itself would become protonated to form a *N*-hydroxy imino cation before attack by $\text{O}_2^{\cdot -}$.⁷² It has been shown that intramolecular H-bond donors increase the reactivity of $\text{O}_2^{\cdot -}$ by disrupting the natural delocalization of its unpaired electron to the non-bonding oxygen, allowing for an increased reactivity towards nitrones.⁵² Calculations of the $\text{O}_2^{\cdot -}$ adduct of **5** with and without explicit H_2O molecules suggest that the peroxy adduct is basic enough to abstract a proton from the amidine NH in both conditions. In each case, the proximal NH is deprotonated (**Figure 1-9**).

Figure 1-9. Optimized structures for **5**, O₂^{•−} adducts, and calculated ΔG_{rxn} , formed from O₂^{•−} with and without explicit H₂O molecules at the PCM/B3LYP/6-31+G(d,p)//B3LYP/6-31G(d) level of theory.

The reaction of **5** with ONOO⁻/ONOOH proved to be very interesting, as an EPR spectrum was obtained for ONOO⁻ with a linear nitron for the first time, and this reaction was shown to completely convert **5** to **6** *in situ*. Gaussian calculations were performed for both the nucleophilic attack and the radical addition of ONOO⁻/ONOOH to PBN and **5**. The two mechanisms were found to have similar ΔG_{rxn} for ONOOH (-15.6 kcal/mol for singlet, and -20.4 kcal/mol for triplet products, respectively) but dramatically different predicted ΔG_{rxn} for ONOO⁻ (-50.5 kcal/mol for singlet, and -6.7 kcal/mol for triplet products, respectively) (**Figure 1-10**). In each case, NO₂ was predicted to be formed and H-bond with the amidine moiety, which may stabilize a transition state between the reactants and adduct formation. While it is not yet clear if these two mechanisms would ultimately yield different products, the predicted ΔG_{rxn} seems to match the observation that a different mechanism predominates in mildly acidic pH, favoring ONOOH formation, than at strongly basic pH. ONOOH is known to exist as a bi-radical trapped in a 'solvent cage' ('OH-NO₂'), and is significantly less stable than ONOO⁻. The observation of an EPR spectrum for both **5** and PBN in the presence of ONOO⁻/HCl suggests that the mechanism at least in part acts by forming triplet products. However, this EPR spectra was not of an O-centered adduct to the nitron, which would resemble an 'OH-spectra, but instead a nitroxyl with no H-splitting, which suggests that either the predicted O-centered radical adduct is very short lived and thus not observed, or decomposition of the nitron occurs before formation of the nitroxyl. This mechanism warrants further investigation, particularly due to the high degree of conversion of **5** to **6** by ONOO⁻/HCl which could be physiologically relevant when studying uncoupled NOS, a known producer of ONOO⁻, as a target.

Figure 1-10. Optimized structures for **5** ONOO[•], ONOOH singlet and triplet adducts, and calculated ΔG_{rxn} at the PCM/B3LYP/6-31+G(d,p)//B3LYP/6-31G(d) level of theory.

Predictive NOS Docking

After *in silico* calculations predicted that **5** would neutralize ROS with ΔG_{rxn} on par or better than that of PBN, the second part of the proposed mechanism of action, NOS inhibition, was also explored *in silico*. The NOS inhibitor moiety chosen for **5/6**, a methyl amidine, is known to act as a permanent inhibitor of NOS through destruction of the active site heme by a heme-oxygenase mechanism to release carbon monoxide and biliverdin.^{70,71} Interestingly, our laboratory has shown that the enzyme heme oxygenase-1 (HO-1), that catalyzes the breakdown of free heme, is beneficial in ischemic stroke⁷³ although it is unclear if methyl amidines affect heme-containing proteins beyond that of the NOS family. The predicted ΔG shown in **Table 1.2** suggest that both **5** and **6** are able to coordinate with the active site Glu at energies that are comparable to that of the native ligand L-arginine and potent iNOS inhibitor 1400W. A closer look at the values suggests some differences in the x-ray crystal structures of L-arginine versus 1400W bound proteins: L-arginine ligand ΔG of binding for L-arginine x-ray crystal structures were very near to 1400W ΔG (e.g. -10.32 kcal/mol compared to -10.37 kcal/mol for L-Arginine and 1400W binding to L-arginine eNOS, respectively) while binding to 1400W x-ray crystal structures produced at least a -1.0 kcal/mol difference (e.g. -9.31 kcal/mol compared to -10.34 kcal/mol for L-arginine and 1400W binding to 1400W eNOS, respectively). This is likely due to differences in the active site conformation of the x-ray crystal structures between the two bound ligands. Because the molecules **5** and **6** more closely resemble the structure of 1400W, the ΔG values from the 1400W-bound NOS isozymes are likely to more closely simulate the true interaction.

The 'pro-drug' **5** was predicted to bind to each NOS isozyme as well or better than the 'active drug' **6**. This is not ideal as it would suggest that the parent molecule could inhibit NOS globally, which was hoped to be avoided by this study. These data need to be confirmed in an *in vitro* binding assay for both ligands. However, the modeling approach taken here could be used to design derivations of **5** and **6** that would cause decreased favorability of binding for the 'pro-drug' form, which would be lost upon reacting with ROS. Such derivations would be made to the *tert*-butyl moiety that may increase bulk and steric hindrance, or increase electrostatic repulsion from the nearby carboxylate of the active site heme.

Synthesis of **5** and Intermediates

Upon favorable *in silico* ROS reactivity and NOS active site docking, the molecule **5** was moved forward for synthesis. The four-step, straightforward synthesis was carried out with little hindrance and a favorable overall yield. Due to the instability of some intermediates, namely the brominated **2** and nitron **5**, reactions were carried out under an argon atmosphere, and products were stored at -20 °C. The *N*-boc benzyl alcohol **1** was activated by Appel bromination using a method optimized in Dr. Schiefer's lab. The separation of **2** was first carried out using a manual column, and afforded 83% yield, however switching to flash chromatography separation increased the recovery to a 91.2% yield. The S_N2 reaction of **2** with *tert*-butylamine was then carried out at room temperature in the presence of K₂CO₃. It was observed that adding **2** in DMF dropwise to a stirring solution of *tert*-butylamine and K₂CO₃ resulted in higher yield of **3** (96.4%) than if *tert*-butylamine was added dropwise to **2** followed by addition of K₂CO₃ (84%). The synthesis of **4** from **3** was performed in two steps: the de-protection of the boc-

protected amine by trifluoroacetic acid, followed by formation of the amidine through an S_N2 reaction with ethylacetimidate as adapted from Fantacuzzi et. al.⁷⁴ **4** was isolated after lyophilization of the ether and EtOAc-washed aqueous layer, as a highly hygroscopic, sticky solid that was insoluble in chloroform and acetone. The formation of the nitron **5** from oxidation of **4** by $\text{NaWO}_4\text{-H}_2\text{O}_2$ was first attempted at 0 °C over 2 hours. This resulted in negligible formation of **5** that was not recovered. Upon increasing both the reaction temperature (0 °C to rt) and the time (2 hr to 12 hr), coupled with more vigorous stirring, **5** was formed at 66.2% yield. Normal phase separation by flash chromatography required high polarity (~ 18% MeOH in CH_2Cl_2) to elute **5**, which suggests that future compounds that are more polar than **5** should be purified by a different method.

EPR Spin Trapping

After characterization by NMR, HPLC, and LC-MS/MS, **5** was taken to the EPR core facility at The Ohio State University Wexner Medical Center for spin-trapping characterization. The goals of these experiments were to first establish that **5** is able to trap free radical species in the same fashion as established nitron spin traps, and to characterize the EPR spectra of known free radicals for future identification of radicals using **5** in unknown systems. **5** was observed to spin trap $\text{O}_2^{\cdot-} / \text{HO}_2^{\cdot}$, $^{\cdot}\text{OH}$ and $^{\cdot}\text{CH}_3$ to yield EPR spectra that strongly resemble those of PBN. Since the amidine moiety is outside of EPR coupling range, it is not surprising that the substitution has a small influence in the hfsc's observed for **5** compared to the 'un-substituted' PBN.

Previous EPR studies with PBN and $\text{ONOO}^{\cdot}$ have been performed, but no direct detection of free radicals have been observed, instead incidental radicals formed from

proteins, uric acid, or dihydropyridines were used for characterization.⁷⁵⁻⁷⁷ In this study however, addition of ONOO⁻ to **5** or PBN resulted in the same three-line EPR spectra with a much larger signal intensity than that of the two other radical systems. A search for literature precedent found a study by Turnbull et. al. revealed a similar three-line spectra from PBN hydrolysis to form a *tert*-butyl nitroso, 2-methyl-2-nitrosopropane (MNP) in the presence of Amyloid- β that was hypothesized to be due to H₂O₂ oxidation of PBN to cause hydrolysis, followed by further oxidation of MNP.⁷⁸ As was described previously, ONOOH exists as a bi-radical, which would be capable of oxidizing the nitron to cause hydrolysis, then further oxidizing the *tert*-butyl nitroso to yield an EPR-detectable species. Importantly, this species would give the same hfsc's for **5** as for PBN, which would correlate to the observed spectra. This hypothesis could be confirmed by performing EPR with *tert*-butyl hydroxylamine in the ONOO⁻ system to observe if the resulting hfsc's match those observed herein. The *in silico* prediction of a **5**-O⁻ or **5**-OH adduct from the reaction with ONOO⁻/ONOOH was not observed by EPR however - such an adduct would yield hfsc's similar to that of the Fe²⁺/H₂O₂ system. This would suggest that either the **5**-O⁻ adduct is highly unstable, and converts very rapidly to the aldehyde species, or that ONOO⁻/ONOOH causes a non-radical decomposition of PBN and **5** to then allow the formation of a *tert*-butyl nitroso radical by oxidation.

HPLC-PDA Analysis of Radical Systems

The formation of benzaldehyde from the decomposition of PBN in the presence of free radicals has been repeatedly shown. To prove that this mechanism was conserved for the nitron **5**, the EPR systems described above were analyzed by HPLC-PDA to confirm the formation of benzaldehyde from PBN, then the proposed molecule **6** from **5**. Multiple

free radicals are known to be formed under ischemic and ischemia/reperfusion conditions,⁷⁹ making it vital to ensure that **6** is formed from **5** for each radical species to ensure its activation at the desired site. Benzaldehyde was formed from PBN in significant quantities for each of the $\text{O}_2^{\cdot -} / \text{HO}_2^{\cdot}$, $\text{Fe}^{2+} / \text{H}_2\text{O}_2$, and $\text{ONOO}^- / \text{HCl}$ systems. These systems were attempted to be analyzed by LC-MS/MS, however, due to the difficulty of ionizing benzaldehyde for detection, such experiments did not produce meaningful data. After PBN decomposition was confirmed, the EPR solutions of **5** were analyzed by HPLC-PDA to observe the formation of a new peak at 3.35 min for each system, a dramatic shift from **5** (pure **5** RT = 5.43 min). This shift in RT was accompanied by a change in λ_{max} , from 289 nm for **5**, to 249 nm for the new peak. The shift in λ_{max} correlates with PBN decomposition, as the nitronyl bond is cleaved. While benzaldehyde exhibits an increase in RT from PBN due to its more-non-polar nature, the decomposition of **5** to **6** would cause an increase in hydrophilic character, such as that seen in the synthetic precursor, **4**. This new peak was observed to be the only peak in the **5**- $\text{ONOO}^- / \text{HCl}$ system, mirroring that of PBN- $\text{ONOO}^- / \text{HCl}$. LC-MS/MS analysis of this solution produced a strong signal corresponding to the molecular weight of **6**. Using this to create an LC-MS/MS MRM analysis method, the same compound was observed in the other radical systems as well. Taken together, these data strongly suggest the formation of **6** after reaction of **5** with physiologically relevant free radicals. These studies will allow rapid screening of future compounds to confirm they exhibit a pro-drug character before proceeding to *in vitro* assays.

Confirmation of **6** Formation

EPR, HPLC-PDA, and LC-MS/MS analysis of the **5**-radical systems show the formation of a more-polar compound with a molecular weight matching that predicted for **6**. However, this system yielded too little **6** for isolation and structural characterization. The conditions for the **5**-ONOO⁻/HCl system were replicated and scaled up after it was found that simply adding ONOO⁻ to **5** in ddH₂O did not yield **6**. This change in reactivity is likely due to the strongly basic solution ONOO⁻ (0.3 M NaOH), which would not allow protonation to the more reactive ONOOH (pK_a = 6.8). Upon using PBS and HCl to lower the pH to 6, ONOO⁻ addition was found to decompose nearly all of **5** to the compound hypothesized to be **6** as observed by HPLC-PDA. Isolation of **6** from the aqueous solution allowed acquisition of ¹H-NMR to observe a peak at 10.0 ppm that confirms the formation of a benzaldehyde while retaining the amidine moiety. The isolation of **6** from the aqueous salt solution was non-trivial due to its high polarity, thus future studies will aim to more efficiently produce and isolate the aldehyde decomposition product from synthesized nitrones by using alternative synthetic routes. The ¹H-NMR confirms that **6** is being formed from free-radical reactions with **5**, and confirms that the LC-MS/MS MRM analysis method for **6** is accurate for the monitoring of **5:6** levels *in vitro* and *in vivo*. Upon confirmation of the proposed *in silico*, synthetic, and *in situ* hypotheses, **5** was advanced for *in vitro* screening.

Experimental

Computational Methods

Ab initio structures of nitrones, radicals, and radical adducts were generated using Avogadro⁸⁰ at the MMFF94 level. Optimized geometries were then determined by density functional theory (DFT)⁸¹ at the B3LYP/6-31G* level of theory as previously

described⁵² yielding no imaginary vibrational frequency. All calculations were performed using Gaussian 09⁸² at the Ohio Supercomputer Center and visualized by GaussView 5.0 software. B3LYP/6-31G* geometries were corrected using a scaling factor of 0.9806⁸³ for the zero-point vibrational energy (ZPE). Solvation effects on the gas-phase calculations were determined using the PCM,^{84,85} and spin and charge densities were assigned using natural population analysis (NPA)⁸⁶ at the PCM/B3LYP/6-31+G** level of theory. All doublet and triplet calculated minima yielded negligible spin contamination ($0.75 < \langle S^2 \rangle < 0.76$). $\Delta G_{\text{rxn},298\text{K}}$ (kcal/mol) of adduct formation was determined by $\Delta G_{\text{Adduct}} - \Delta G_{\text{Reactants}}$.

Protein docking studies were carried out using Swissdock.ch^{54,55} and visualized by UCSF Chimera software.⁸⁷ X-ray crystallographic structures of each NOS isoform with L-arginine or 1400W bound were used to determine the free energy of interaction with the enzyme active site. Docking was limited to ± 10.0 Å of the native bound ligand (L-arginine or 1400W) in the X, Y, and Z directions.

Synthesis

Materials were purchased from commercial suppliers (Sigma or Fisher), and used as received. Each synthetic reaction was carried out under an Argon atmosphere, and products were stored at -20 °C after purification. Reactions were monitored by TLC (LuxPlate silica gel 60 F254 plates), and revealed by UV (254 nm), iodine, or ninhydrin stains. Flash column chromatography was performed using a Teledyne Combiflash R_f with RediSepR_f Gold columns. Purity analysis by HPLC was performed using a Shimadzu Prominence HPLC (LCD-20AD) with temperature controlled autosampler (SIL-20AC), and PDA (SPD-M20A) detector. HPLC conditions: Phenomenex Kinetix[®]

core column (2.6 μm , C18, 100 $\text{\AA}$, 100 x 4.6 mm), mobile phase A = 25 mM ammonium formate in water (0.1% formic acid [FA]), mobile phase B = ACN (0.1% FA), 1.0 mL/min, 5% B to 95% B over 5 min followed by 95% B for 2 min then 5% B for 5 min re-equilibration (total run time = 12 min). ^1H and ^{13}C NMR were taken using a Bruker Avance 600 MHz spectrometer (cryoprobe), and HRMS was acquired using a Waters Synapt high definition mass spectrometer (HDMS) equipped with a nano-ESI source positive mode.

tert-butyl (3-(bromomethyl)benzyl)carbamate (**2**): *tert*-butyl (3-(hydroxymethyl)benzyl)carbamate (**1**, AstaTech, 500 mg, 1.0 eq) was dissolved in 8.8 mL anhydrous CH_2Cl_2 at 0 $^\circ\text{C}$ with stirring. CBr_4 (838 mg, 1.2 eq) was added spatula-wise over 5 min, followed by PPh_3 (663 mg, 1.2 eq) spatula-wise over 10 min. Reaction was let warm to rt and let stir for 30 hr. CH_2Cl_2 (50 mL) was added, washed with brine (3 x 20 mL), evaporated, and purified by column chromatography (hexanes:EtOAc). **2** was afforded as a clear solid (577 mg, 91.2%), ^1H NMR (CDCl_3 , 400 MHz): δ 7.35-7.24 (4H, m), δ 4.88 (1H, br), δ 4.51 (2H, d, J = 3.92 Hz), δ 4.34 (2H, d, J = 3.60 Hz), δ 1.49 (9H, s).

tert-butyl (3-((*tert*-butylamino)methyl)benzyl)carbamate (**3**): To a stirring solution of *tert*-butylamine (237 mg, 1.8 eq) and K_2CO_3 (234 mg, 1.3 eq) was added **2** (542 mg, 1.0 eq) in 3.6 mL anhydrous DMF by syringe at rt. The reaction was let stir for 3 hr, then diluted with 15 mL dH_2O , and extracted with ether (3 x 30 mL). The organic layer was washed with brine (3 x 20 mL), dried with Na_2SO_4 , filtered and evaporated. Separation by column chromatography (hexane:EtOAc) yielded **3** as a white solid (509 mg, 96.4%),

^1H NMR (CDCl_3 , 400 MHz): δ 7.31-7.25 (3H, m), δ 7.18-7.17 (1H, m), δ 4.85 (1H, br), δ 4.32 (2H, d, J = 3.68 Hz), δ 3.73 (2H, s), δ 1.49 (9H, s), δ 1.20 (9H, s).

N-(3-((*tert*-butylamino)methyl)benzyl)acetimidamide (**4**): **3** (390 mg, 1.0 eq) was dissolved in 44 mL CH_2Cl_2 with stirring at 0 °C. Trifluoroacetic acid (10.2 mL, 100 eq) was rapidly added by syringe, and let stir for 2 hr on ice. The solvent was evaporated to give a brown oil which was re-suspended in 1.5 mL 1:1 dH_2O /EtOH, and 2 N NaOH was added until pH > 12. The mixture was extracted with CH_2Cl_2 (2 x 15 mL), dried with Na_2SO_4 , filtered, and evaporated in a round-bottom flask. The residue was dissolved in 5 mL anhydrous EtOH, ethyl acetimidate (165 mg, 1.0 eq) in 2 mL EtOH was added dropwise and let stir at rt for 12 hr. The reaction was evaporated, re-suspended in 15 mL dH_2O , and then the aqueous layer was washed with ether (3 x 15 mL) and EtOAc (2 x 15 mL). Lyophilization of the aqueous layer gave **4** as a white, hygroscopic solid (295 mg, 94.7%), ^1H NMR (CD_3OD , 400 MHz): δ 7.52 (2H, s), δ 7.47-7.46 (2H, m), δ 7.39-7.37 (1H, m), δ 4.53 (2H, s), δ 3.98 (2H, s), δ 2.32 (3H, s), δ 1.38 (9H, s).

N-tert-butyl- α -(3-[ethanimidamidomethyl]phenyl)nitron (**5**): **4** (174 mg, 1.0 eq) was dissolved in 2 mL MeOH, and added to a round-bottom flask containing $\text{Na}_2\text{WO}_4(\text{H}_2\text{O})_2$ (10 mg, 0.05 eq) at 0 °C with stirring. Ice-cold 30% H_2O_2 (263 μL , 4.0 eq) was added dropwise over 5 min. The reaction was let warm to rt and let stir vigorously for 12 hr. 20 mL CH_2Cl_2 was added, the organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (2 x 20 mL). Purification of the organic layer by column chromatography (CH_2Cl_2 :MeOH) afforded **5** as a brown oil (122 mg, 66.2 %), ^1H NMR ($\text{DMSO}-d_6$, 400 MHz): δ 9.89 (1H, br), δ 9.29 (1H, br), δ 8.84 (1H, br), δ 8.60 (1H, s), δ 8.12 (1H, d, J = 5.24 Hz), δ 7.89 (1H, s), δ 7.47-7.39 (2H, m), δ 4.49 (2H, s), δ 2.22 (3H, s), δ 1.52

(9H, s); ^{13}C NMR (DMSO- d_6 , 100 MHz): 164.75, 135.65, 132.39, 129.43, 128.99, 128.91, 128.75, 127.54, 71.05, 45.69, 28.32, 19.22; HPLC purity (254 nm): 95.8%; HRMS (M + H) m/z 248.1759, calcd for $\text{C}_{14}\text{H}_{21}\text{N}_3\text{O}$ 248.1757.

N-[*(3-formylphenyl)methyl*]ethanimidamide (**6**): To a stirring solution of **5** (5.0 mg, 1.0 eq) in 785 μL PBS was added 20 μL 6 N HCl at 37 °C. Peroxynitrite (200 μL of a 50 mM solution in 0.3 M NaOH, 0.5 eq) was added in 50 μL aliquots over 5 min. The reaction was let stir for 1 hr and monitored by HPLC-PDA. The solution was extracted with CH_2Cl_2 (2 x 0.5 mL) and the aqueous fractions were pooled then lyophilized. Addition of ACN (1.0 mL) to the white solid followed by filtration allowed separation from the insoluble salts. Lyophilization afforded **6** as a white solid (1.2 mg, 33.7 %), ^1H NMR (CD_3OD , 400 MHz): δ 10.04 (1H, s), δ 7.93-7.95 (2H, m), δ 7.71-7.65 (2H, m), δ 4.58 (2H, s), δ 2.30 (3H, s)

EPR Spectroscopy

EPR spectra were collected at ambient temperature with microwave power 12.6 mW, frequency 9.86 GHz, receiver gain 1.0×10^5 or 1.0×10^4 , modulation amplitude 1 G, scan time 20.48 sec, time constant 40.96 msec, and sweep width 120 G. Phosphate buffered saline (PBS) used for EPR was supplemented with diethylenetriaminepentaacetic acid (DTPA, 100 μM). ROS-generating solutions were rapidly added to solutions of indicated spin trap and taken up in 50 μL glass capillary tubes for EPR spectral acquisition. For HPLC-PDA analysis, solutions were allowed to incubate for 24 hr at 37 °C, then diluted 1:1000 in 95:5 mobile phase A:B, and analyzed as above. Benzaldehyde (Sigma) was run separately as a control to compare to PBN-ROS systems and confirm PBN decomposition.

O₂^{•-}/HO₂[•]-generating system: A 50 µL final 20% PBS/DMSO solution of 35 mM **5** or PBN contained 10 µL saturated KO₂ DMSO.

•OH/CH₃-generating system: A 50 µL final 30% PBS/DMSO solution of 35 mM **5** or PBN contained 90 mM FeSO₄ and 1% H₂O₂.

ONOO⁻/HCl system: A solution of ONOO⁻ in 0.3 M NaOH (Cayman Chemical) was determined to be 50 mM by absorbance at 302 nm using the literature extinction coefficient (1670 M⁻¹cm⁻¹). A 50 µL final 30% PBS/DMSO solution of **5** or PBN contained 10 mM ONOO⁻ at pH~6 (HCl).

Chapter 2

5 as a Hit Molecule – ROS-Induced Formation of 6

Introduction

Similar to whole animal models, *in vitro* systems yield conflicting results for the effects of NOS inhibition, which seems to either potentiate⁸⁸ or protect from⁸⁹ oxygen-glucose deprivation (OGD)-induced neuronal death. NO is known to affect various pathways in a concentration-dependent manner.⁹⁰ NO has been shown to both promote activation of the pro-survival Protein Kinase B (Akt),⁹¹ and be produced by the Phosphatidylinositol-4,5-bisphosphate 3-Kinase (PI3K)/Akt/NOS pathway.⁹² Mitochondrial-induced NO production by activation of nNOS provides neuroprotection from lethal stress and promotes vasodilation, which was abolished by a selective nNOS inhibitor.⁹³ During OGD, NO-stimulated Extracellular Signal-Regulated Kinases 1/2 (ERK1/2) provide neuroprotection through increased Superoxide Dismutase (SOD) expression, which was similarly abolished by NOS inhibition.⁹⁴ Yet it is also observed that inhibition of NOS activity provides neuroprotection and improved mitochondrial function after OGD-exposed cultured neurons through the ERK1/2 pathway.⁹⁵ Alternatively, the role of NOS in inflammation is unequivocal, and the inhibition of iNOS under pathological conditions results in reducing its toxic overproduction of NO.⁹⁶

Taken together, it appears that the protective potential of NOS in ischemic tissue is dependent on its contribution to the NO/ROS balance, likely through its uncoupling and/or local environment. The translation of neuroprotection by NOS inhibitors *in vitro* to *in vivo* is difficult to assess as NO derived from eNOS in vascular endothelial cells is protective to neurons,⁹⁷ and cross-talk between the isoforms impacts NOS uncoupling.⁹⁸ Thus single-cell type cultures may not provide an accurate portrayal of their effects in whole tissue.

The clinically tested nitrone NXY-059 provided neuroprotection to primary neuronal cultures exposed to OGD, while recently developed nitrones like the quinolinylnitron RP19 achieve the same level of protection at 100-fold lower concentrations.³⁹ Interestingly, NXY-059 was not neuroprotective in a model of intracellular oxidative stress whereas other antioxidants showed activity; therefore NXY-059 is thought to primarily act extracellularly rather than intracellularly to confer neuroprotection.⁹⁹ PBN, one of the first and most-basic nitrones, requires doses as high as 1 mM to provide neuroprotection to OGD-exposed neurons.¹⁰⁰ Increasing the hydrophilicity with hydroxyphenyl PBN-type derivatives gives increased antioxidant capacity and is shown to reduce nitrotyrosine formation due to their structural similarity to tyrosine.³⁸ These nitrones showed dose-dependent protection of SH-SY5Y cells from oxidative stress at concentrations as high as 500 μ M, and a decrease in nitrite production in lipopolysaccharide (LPS)-treated primary microglia and astrocytes. In some cases, high concentrations of nitrones have been observed to have a minimal effect on neuronal viability after OGD yet provide significant reductions in markers of oxidative stress.¹⁰¹

For this reason there has been increased focus on the effect of nitrones on pro-survival, anti-inflammatory, and anti-apoptotic pathways.

The nitrone TBN increased axonal outgrowth in cultured neurons, which was determined to be through the PI3K/pAkt/phospho-glycogen synthase kinase 3 beta (pGSK-3 β) pathway.¹⁰² A model of chronic cerebral hypoperfusion caused increased oxidative stress and pro-apoptotic signaling pathways such as B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), and caspase-3, and decreased Akt and GSK-3 β activation. These detriments were ameliorated by TBN treatment, which was reversed by the PI3K inhibitor LY29004. While Akt activation has been observed with other nitrones as well, the mechanism is not fully elucidated, however the pro-survival properties of the PI3K/Akt pathway under oxidative stress have been long understood.¹⁰³ Nitrones have also been shown to possess anti-inflammatory activity, which may further contribute to their beneficial effects *in vivo*. The anti-inflammatory properties of PBN in rat white matter neurons subjected to ischemia were linked to decreased interleukin-1 beta (IL-1 β), tumor necrosis factor alpha (TNF- α), and iNOS expression,¹⁰⁴ and a reduction in CD8+ T-cell infiltration.¹⁰⁵ While the individual effects of nitrones and NOS inhibitors on pro-survival and anti-inflammatory signaling have been studied, it is unclear what the overall effects would be of a combination nitrone-NOS inhibitor such as **5/6**. Additionally, while *in silico* calculations predict NOS inhibition by both **5** and **6**, confirmation in an *in vitro* assay is necessary to support the claim that the molecules proposed are indeed NOS inhibitors.

Results

In vitro Neuroprotection Assay

5 was tested in an *in vitro* model of ischemia/reperfusion, OGD with 24 hr reperfusion, to assess its neuroprotective potential. A dose-finding study was first performed to determine the optimal concentration. SH-SY5Y cells exposed to 1.5 hr OGD treated with increasing **5** or PBN (from 0.05 μ M to 100 μ M) were assayed for viability by PrestoBlue and plotted as ratio to vehicle control (**Figure 2-1**). PrestoBlue is an NADPH-converted resazurin dye that was found to give reproducible measurements of viability on par with that of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) at higher throughput (data not shown). A bi-phasic trend was observed for **5** treatment, with increasing viability from 0.1 μ M to 1.0 μ M (1.21 to 2.57 times greater than vehicle; $p < 0.05$, $p < 0.01$ respectively, $n = 6$), giving way a decline to baseline at 100 μ M. PBN trended in a similar fashion, but did not approach significance at any concentration tested. Previous studies with PBN found that effective doses for OGD-treated primary neurons ranged from 100 μ M to 1 mM, and afforded ~50% increased viability.¹⁰⁰ In the present study, ~50% increased viability was observed for 10-100 μ M PBN in SH-SY5Y cells, but was not significant due to the high variability of the assay. 1.0 μ M **5** showed significantly greater neuroprotection than PBN at the same dose ($p < 0.05$). 1.0 μ M was thus determined to be the optimal dose of **5**, and was used in further *in vitro* studies to elucidate its protective mechanisms.

Figure 2-1. *in vitro* dose-response plot of SH-SY5Y viability after oxygen-glucose deprivation (OGD) and 24 hr treatment relative to vehicle control. **5** showed the highest neuroprotection at 1.0 μM, which was significantly higher than that of 1.0 μM PBN (n = 6, $p < 0.05$). Data represented as mean $\pm$ SEM, * $p < 0.05$, and ** $p < 0.01$, from vehicle control, One-way ANOVA followed by Newman-Keuls *post hoc* test.

Effect of **5** on Pro-Survival and Anti-Apoptotic Signaling Pathways

The mechanisms of the neuroprotection afforded by **5** were investigated by Western blotting (**Figure 2-2**). SH-SY5Y cells subjected to OGD and treated 24 hr with 1.0 μ M **5** showed a dramatically increased phospho-Akt/total Akt ratio (4.1 times higher, $p < 0.05$). This increase was also observed for PBN, albeit each to a lesser extent than **5** alone, and was not affected by the addition of the NOS inhibitor 1400W (1.0 μ M). Despite the increase in pAkt for PBN treatment, the high variability imparted by OGD treatment did not yield significance at $n = 6$. The ratio of ERK1/2 phosphorylation was increased after OGD exposure, and appeared to be further heightened by **5**, however this did not approach significance. Treatment with **5** also showed a reduction in OGD-induced protein nitration. 3-Nitrotyrosine (3-NT) is formed due to direct nitration of protein tyrosines by ONOO^- , and is a marker of oxidative and nitrosative stress. 3-NT was increased by OGD 1.5-fold in vehicle groups, and ameliorated by 48% upon treatment with **5** ($p < 0.01$). Direct addition of ONOO^- to SH-SY5Y cells dramatically increased 3-NT formation, yet treatment with **5** was not able to significantly reduce this insult despite a downward trend. Similarly, the pro-apoptotic cleavage of caspase-3 was induced by OGD, but was significantly reduced with **5**-treatment compared to vehicle (47% reduction, $p < 0.01$). Despite the dual nature of ROS-scavenging and NOS-inhibition, **5** was able to retain the protective effects on signaling pathways as was observed for conventional nitrones.

Figure 2-2. Western blot analysis of SH-SY5Y cells exposed to OGD and 24 hr treatment. (A) 5 treatment (1.0 μ M) was found to increase pAkt/Akt ratio ($n = 6$), and (B) decrease total 3-NT and (C) cleaved caspase-3 and (D) pERK1/2 compared to vehicle control ($n = 3$). Data represented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, from respective vehicle controls, One-way ANOVA followed by Newman-Keuls *post hoc* test.

Anti-Inflammation and iNOS Inhibition of **5** & **6**

Microglia express iNOS and convert to an inflammatory phenotype upon stimulation with LPS, leading to an overproduction of NO which reacts with oxygen to ultimately form nitrite. 1400W is a potent iNOS inhibitor that has been previously shown to exhibit anti-inflammatory properties.¹⁰⁶ To investigate the anti-inflammatory potential of **5** and/or **6**, SIM-A9 microglia were stimulated with LPS (100 ng/mL), and treated with vehicle or drug for 24 hr. Nitrite, as measured by Griess assay, was increased as expected after LPS stimulation, and attenuated by 1.0 μ M 1400W to levels near that of sham cells (60% decrease from vehicle, $p < 0.001$; **Figure 2-3**). **5** was not observed to lower NO production at concentrations as high as 50 μ M, although the highest dose appeared to cause a slight decrease. Doses from 1.0 μ M to 50 μ M of **6** exhibited a dose-dependent decrease in nitrite (from a ~19% to ~33% decrease, $p < 0.01$ and $p < 0.001$, respectively). PBN was not observed to cause a reduction in nitrate at the same concentrations (data not shown). This indirect measurement of NO production suggests a reduction in iNOS activity due to **6** treatment but not **5**, which indicates that **5** needs to be converted to **6** to allow iNOS inhibition. This assay can be improved however, by directly measuring the conversion of L-arginine to L-citrulline by NOS, as **5** decomposition itself causes the formation of NO which would cause an inaccurate assessment of iNOS activity.

Figure 2-3. Griess assay of LPS-stimulated SIM-A9 cells following 24hr treatment indicates anti-inflammatory potential for **6** and 1400W, but not **5** ($n = 6$). **6** treatment, but not **5** treatment, decreased nitrite in a dose-dependent manner which suggests iNOS inhibition. Data represented as mean $\pm$ SEM, ** $p < 0.01$, and *** $p < 0.001$, from vehicle control, One-way ANOVA followed by Newman-Keuls *post hoc* test.

OGD-Induced Formation and Localization of **6** from **5**

5 was observed to be converted to **6** *in situ* in the presence of oxygen-centered free radicals as measured by EPR and HPLC-PDA. It was then hypothesized that this decomposition would occur *in vitro*, and that **6** would be formed at higher levels in cells exposed to ischemia/reperfusion conditions. SH-SY5Y cells exposed to OGD and treated for 24 hr with 10 μ M **5** as before were used to measure the extracellular and intracellular formation of **6**. Medium of sham and OGD-exposed, **5**-treated cells and their respective cell lysates were worked up and analyzed by LC-MS/MS using the MRM methods developed previously. The AUC for **6** was normalized between samples by the internal standard, then divided by the intra-run AUC for **5** to express levels of **6** as a ratio of **5** (**Figure 2-4**). The supernatant fractions were found to have comparable ratios of **6:5** for both sham and OGD-exposed cells. **6** was found to be formed in considerably higher levels in the intracellular fractions of OGD-exposed cells compared to sham cells (129% and 29% **6:5** AUC, respectively, $p < 0.001$). Due to its high polarity, **6** may be formed by intracellular ROS from **5**, but not be able to diffuse out, leading to localization within ROS-stressed cells. These data support the claim that **5** is cell permeable, and that **6** will be preferentially formed in cells undergoing ischemia. Taken together, the *in vitro* data show that **5** is a cell permeable, neuroprotective nitron that forms the anti-inflammatory **6** upon ischemia/reperfusion. This neuroprotection is potentially due to its upregulation of pAkt, and decrease of 3-NT and cleaved Caspase-3 levels.

Figure 2-4. Levels of **6** with respect to intra-run **5** in supernatant and lysate fractions of sham and OGD-exposed SH-SY5Y cells treated with **5** as measured by LC-MS/MS. **6** was observed to be formed at comparable levels in the supernatant, but at considerably higher levels in the lysate of OGD-exposed cells compared to sham cells. AUC normalized between samples with IBA internal standard. Data represented as mean $\pm$ SEM, $n = 3-6$, *** $p < 0.001$, from respective sham control, One-way ANOVA followed by Newman-Keuls *post hoc* test.

Discussion

5 as a Hit Molecule – ROS-Induced Formation of 6

Protein docking of both **5** and **6** to each NOS isoform suggests a similar affinity for the protein active sites for both compounds. While this needs confirmation in an *in vitro* assay, future compounds will ideally possess reduced affinity of the nitronone prodrug for NOS compared to its decomposition product. After synthesis of **5**, EPR spectra were acquired of radical adducts which confirm its antioxidant potential. Direct NO measurement was not performed in the ROS systems herein; however, NO formation from PBN was confirmed to be due to nitronone decomposition in similar systems as well as both *in vitro* and *ex vivo* by EPR using ^{15}N -PBN.¹⁰⁷ Furthermore, analysis of these systems by HPLC-PDA and LC-MS/MS show the formation of **6** in each of the ROS systems.

***In vitro* Dose-Dependent Neuroprotection of SH-SY5Y Cells by 5**

The antioxidant properties of **5** were further explored through neuroprotection of SH-SY5Y cells exposed to OGD. Human neuroblastoma SH-SY5Y cells express nNOS constitutively,¹⁰⁸ whereas PC12 cells require transfection,¹⁰⁹ which makes SH-SY5Y cells a better model for exploring the particular neuroprotective potential of **5**. The OGD model of ischemia/reperfusion is known to induce oxidative stress in neuronal cultures through the production of $\text{O}_2^{\cdot-}$,¹¹⁰ $\text{ONOO}^{\cdot-}$,^{111,112} and $\cdot\text{OH}$.¹¹³ These ROS were shown above to be trapped *in situ* by **5**, and each were observed to cause decomposition to **6** to some degree. Due to the previously mentioned conflicting findings for NOS inhibition under OGD-induced stress, and the orders of magnitude differences in protection afforded by synthetic nitrones, it was vital to establish neuroprotective

potential of **5** and an optimal dose before exploring its effects on signaling pathways. The nitrone **5** was observed to afford dose-dependent neuroprotection to OGD-exposed SH-SY5Y cells in the low micromolar range. The viability increase was observed to reach a maximum at 1.0 μ M of **5** before declining back down to baseline at higher concentrations. It should be noted that the highest dose of **5** (100 μ M) was not observed to be toxic, but rather non-neuroprotective, as the viability was found to be similar to that of vehicle treated cells. The mechanism of this decline to baseline is uncertain, however it could potentially be due to a large amount of NO release from **5** that could outweigh the benefits of ROS trapping and NOS inhibition. NO is observed to affect different pathways at increasing concentrations,⁹⁰ and high levels are cytotoxic.¹¹⁴ Further studies are required to elucidate this return to the ‘no effect level’. That **5** was significantly more neuroprotective at 1.0 μ M than PBN suggests the amidine substitution of **5** provides some additional beneficial effect, potentially through NOS inhibition or increased reactivity toward ROS through the intramolecular H-bonding predicted above.

Effect of 5 on Neuroprotective and Anti-Apoptotic Mediators

Western blotting was used to investigate the signaling mechanisms of the neuroprotection afforded by **5** *in vitro*. The pro-survival pAkt(Ser473) and pERK1/2 (Thr202/Tyr204) were found to be increased in **5**-treated SH-SY5Y cells exposed to OGD. Akt is a serine/threonine kinase involved in cell proliferation and apoptosis that becomes phosphorylated at multiple potential phosphorylation sites upon binding PIP₃ produced by PI3K. This pathway has been shown to be affected in neurons by both antioxidants¹¹⁵ and NO-donors¹¹⁶ in ischemia. PBN was also observed to elicit an increase in Akt phosphorylation, although not significant at the dose selected (1.0 μ M),

and was unaffected by co-treatment with 1400W. NXY-059 was found to increase pAkt/Akt levels in brains of animals after transient MCAO specific to the infarct and penumbra areas.¹¹⁷ While the authors attributed this to cell surface interactions, it is more likely to be due to increased reactivity of the nitron in those areas, leading to ROS neutralization and NO release. This hypothesis is supported by the data herein which shows greater decomposition of **5** under oxidative conditions *in vitro* analogous to that of the ROS chemical systems. Utilizing this site-specific property can unlock new avenues of drug targeting in ischemia and various pathologies characterized by oxidative stress.

3-NT and cleaved caspase-3 were decreased by 1.0 μ M **5**, which illustrate reductions in ONOO⁻ production and apoptosis induced by OGD. 3-NT has been shown to be formed from the direct oxidation of protein tyrosines by ONOO⁻, and was recently shown to be almost completely though uncoupled NOS¹¹⁸ as opposed to separate mechanisms for O₂⁻ and NO production. The formation of 3-NT has been detected in both ischemia/reperfusion¹¹⁹ and inflammatory pathologies.¹²⁰ In each of these studies, it was found that NOS was directly involved in 3-NT production, and NOS inhibition was able to decrease its formation. The physiological role for 3-NT formation is not yet fully understood, but it appears to be the main mechanism of ONOO⁻ mediated damage as it was found that supplementing cells with peptides containing tyrosines susceptible to nitration protects from ONOO⁻ induced apoptosis.¹²¹ The decrease in 3-NT formation observed here could be due to neutralization of ONOO⁻ by **5**, or to the prevention of its production through inhibiting uncoupled NOS. The mechanism of caspase-3 cleavage and activation is more clearly elucidated: activation of either the intrinsic or extrinsic cell death pathway causes cleavage of procaspase-3 to the active 17 kDa and 12 kDa subunits

that hydrolyze aspartate-containing peptides. Cytochrome c release from the mitochondria induces the intrinsic cell death pathway via caspase-9, which then cleaves caspase-3 to continue the pro-apoptotic signal. NOS has been shown to be involved in caspase-3 dependent apoptosis in ischemia^{122,123} and its inhibition was found to be beneficial, yet basal NO levels were shown to reduce caspase-3 cleavage.¹²⁴ Since oxidative stress is an inducer of caspase-3 cleavage via activation of the intrinsic cell death pathway it can be suggested that uncoupled NOS, not functional NOS, is the primary inducer of caspase-3 in these paradigms. Correction of the ROS/NO imbalance by **5** & **6** through anti-oxidation and uncoupled NOS inhibition after ischemia and reperfusion is proposed to underlie the observed increased Akt phosphorylation, and reduced ONOO⁻ production, 3-NT formation, and caspase-3 cleavage.

Anti-Inflammatory Properties of Decomposition Product 6

Ischemic stroke causes the progression of microglia, the resident macrophages of the central nervous system, toward a pro-inflammatory phenotype that peaks at 72 hr post-ischemia.¹²⁵ This activation causes the production of pro-inflammatory cytokines and free radicals like NO via iNOS, and O₂⁻ via NADPH oxidase. These products are toxic to other resident cells, however the increased expression of MnSOD in activated microglia provides protection from their own ROS generation.¹²⁶ LPS stimulates the toll-like receptor 4 (TLR4) receptor on the surface of microglia to provoke an inflammatory response and induce both iNOS and MnSOD expression.¹²⁷ In the SIM-A9-LPS system, while iNOS is induced, nitrones like PBN (at millimolar levels) are shown to reduce the expression of iNOS under such inflammatory conditions, but not affect its activity.¹²⁸ **5** alone did not affect nitrite production, while **6** showed a dose-dependent reduction, which

strongly suggests iNOS inhibition. Thus, while *in silico* calculations predict similar iNOS affinity for **5** and **6**, only the latter was observed to exhibit inhibition. This provides support to the hypothesis that **5** needs to be converted by ROS to act as a functional NOS inhibitor. The conversion of **5** to **6** was not measured in this system, yet it was initially thought that some decomposition would occur, and thus cause decreased nitrite formation, albeit to a lesser extent than **6** alone. However, due to concomitant upregulation of the far-superior radical scavenger MnSOD, it is likely that insufficient amounts of **5** were reacted to form **6**. Another possible explanation for the lack of nitrite reduction by **5** treatment could be that any conversion of **5** to **6** releases NO which would add to the overall nitrite measured. Thus while iNOS inhibition could have occurred to some extent in **5**-treated activated microglia, the NO created from **6** formation could have hidden this result. These hypotheses can be tested by measuring **5** decomposition and **6** conversion by LC-MS/MS as above, and the extent of iNOS induction after **5** and **6** treatment by Western Blotting. Alternatively, ^{15}N -L-arginine or ^{15}N -**5** could be used to measure ^{15}N -nitrite production by GC-MS to differentiate nitrite derived from iNOS and nitron decomposition, respectively.¹²⁹ Future studies will aim to delineate the *in vitro* antioxidant and NOS inhibition effects of **5** & **6** to further elucidate the mechanisms of neuroprotection and anti-inflammation.

6 Forms Preferentially Under Conditions of Oxidative Stress

The formation of **6** from the reaction of **5** with physiologically relevant ROS was confirmed above *in situ* by HPLC-PDA, LC-MS/MS, and finally by isolation of **6** to allow ^1H -NMR acquisition. Whether this decomposition occurs in living systems was investigated in OGD-exposed SH-SY5Y cells. The development of an MRM method by

LC-MS/MS fragmentation of **6** allowed for the sensitive detection of its formation *in vitro*. Unfortunately, sufficient quantities of **6** for the generation of a standard curve to allow absolute quantitation were not isolated. Thus the LC-MS/MS AUC for **6** was divided by the intra-run AUC for **5** to give a ratio of **6:5** for each sample. This method assumes approximately equal loss of both **5** and **6** during the work-up, which may be an incorrect assumption. However, the AUCs of both **5** and **6** were normalized between samples by using 4(1*H*-Imidazol-1-yl)benzaldehyde (IBA, Sigma) as an internal standard. IBA, bearing a benzaldehyde and an H-bonding imidazole group, was chosen as a standard due to its chemical similarity to **5** and **6** to estimate loss of the latter during sample work-up. Future studies will aim to utilize more appropriate internal standards such as deuterated, ¹⁴C, or ¹⁵N-substituted isomers of the investigated compounds. Nonetheless, the decomposition product **6** was detected *in vitro* by LC-MS/MS in the supernatant and cell lysate of SH-SY5Y cells exposed to OGD. After OGD, levels of **6** were observed to be comparable in supernatant fractions (18.6% and 7.88% of **5** AUC for OGD and sham, respectively), but significantly higher in the lysate of OGD-exposed cells (129% vs. 29% of **5** AUC in OGD and sham, respectively, *p* < 0.001). This result further confirms the hypothesis that **5** would be preferentially converted to **6** in ischemic tissue, very likely due to its reaction with and neutralization of ROS. Coupled with the reduction in nitrite observed in activated microglia by **6**, these data clearly illustrate the ROS-dependent activation of the nitron **5**, which then decomposes to release NO and yield the NOS inhibitor **6** preferentially at the site of oxidative stress induced by ischemia (**Figure 2-5**).

Figure 2-5. Proposed scheme for the neuroprotection afforded by **5** (NOS Inhibitor-Nitrone) through its decomposition to **6** (NOS Inhibitor) to cause inhibition of uncoupled NOS in the presence of ROS generated during ischemia.

Experimental

Cell Culture

Human neuroblastoma SH-SY5Y cells (ATCC) and SIM-A9 mouse microglia (a generous gift from Dr. Kumi Nagamoto-Combs at the University of North Dakota) were separately cultured on poly-D-lysine coated plates in 1:1 Dulbecco's Modified Eagle Medium / Ham's F12 (DMEM/F12) medium containing 5% fetal bovine serum, 5% horse serum and penicillin-streptomycin. Cultures were stored in a 37 °C incubator with a 5% CO₂ atmosphere. Cells were allowed to adhere overnight prior to subjection to experimental systems. 1 x 10⁶ cells SH-SY5Y cells on 96-well plates were used for OGD experiments, and SIM-A9 cells were plated at a density of 1 x 10⁶ on 24-well plates for Griess experiments.

Oxygen-Glucose Deprivation (OGD)

Growth medium was removed from SH-SY5Y cells and replaced with two changes of sterile HBSS (140 mM NaCl, 3.5 mM KCl, 0.4 mM KH₂PO₄, 5 mM NaHCO₃, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 20 mM HEPES, pH 7.4, bubbled with 95%:5% N₂/CO₂), and placed in an air-tight container subsequently purged three times with 95%:5% N₂/CO₂. The container was then placed in a 37 °C incubator for 1.5 hr, with rotation every 30 min. After OGD, the cells were returned to a normoxic environment, and medium was replaced with growth medium and vehicle (PBS) or treatment for 24 hr to simulate reperfusion. Sham cells were left in growth medium under a normoxic environment for the duration of the experiment. Viability was measured by adding 10% PrestoBlue[®] Cell Viability Reagent (Thermo Fisher) for 30 min, and reading fluorescence at 560 nm/590 nm excitation/emission. Viability is reported as a ratio to

vehicle-treated control, and readings result from separate OGD experiments (e.g. n = 6 was from 6 different OGD assays). The experimental furoxan IS-1-41 was used as a positive control for OGD viability due to its reproducible protection in this paradigm.

Western Blotting

SH-SY5Y cells exposed to 1.5 hr OGD followed by 24 hr vehicle or treatment were harvested and lysed by RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Na Deoxycholate, 0.1% SDS, 50 mM Tris, 5 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 1 mM sodium orthovanadate, 50 mM NaF, 10 mM sodium pyrophosphate, protease inhibitor cocktail [Thermo Fisher]). Equivalent amounts of protein as determined by Bradford assay were loaded onto 4-20% tris-glycine gels and separated by electrophoresis. Proteins were transferred to a PVDF membrane, blocked with 5% BSA-TBST, and probed with primary antibodies in blocking buffer (anti-phospho-Akt(Ser473) 1:2000; anti-Akt 1:1000; anti-Nitrotyrosine 1:1000; anti-cleaved Caspase-3(Asp175) 1:1000, anti-phosphoERK1/2(Thr202/Tyr204) 1:2000; anti-ERK1/2 1:1000, Cell Signaling; anti-GAPDH 1:2000, Thermo Fisher) followed by secondary antibody (HRP goat anti-rabbit IgG 1:10,000, Jackson) in 5% milk-TBST. Images were acquired using a BioRad ChemiDocTM XRS+, and densitometry was analyzed using ImageJ software (NIH) normalized to GAPDH loading control.

Griess Assay

SIM-A9 cells were treated with 100 ng/mL LPS (Sigma) along with vehicle or drug treatment for 24 hr. Conditioned medium was then diluted 1:1 with Griess reagent (0.4% naphthylethylenediamine dihydrochloride, and 4% sulphanilamide in 10%

phosphoric acid) prepared fresh, and analyzed by colorimetry at 540 nm after 5 min incubation. Nitrite levels were reported as a ratio to LPS-treated, vehicle control.

LC-MS/MS

Synthesized **5**, and purchased 4(1*H*-Imidazol-1-yl)benzaldehyde (IBA, Sigma) were used to determine LC-MS/MS fragmentation. Isolated **6** from the **5**-ONOO⁻/HCl system was used for **6** fragmentation. MRM analysis of IBA (*m/z* 173.10 → 91.10, 104.10, 117.05; RT: 4.13 min), **5** (*m/z* 248.20 → 148.00, 134.15, 190.05; RT: 4.81 min), and **6** (*m/z* 177.00 → 119.10, 90.95, 65.15; RT: 3.87 min) were determined. A standard curve for **5** was generated using pure samples applied to blank matrix (0.01 μM, 0.1 μM, 0.5 μM, 1.0 μM, and 5.0 μM; $R^2 = 0.99$).

In vitro supernatant samples were spiked with internal standard (200 μL 500 nM IBA in 2% FA) and used as collected, while cells were detached by trypsin, centrifuged (14k rpm, 5 min, 4 °C) to isolate the pellet, then lysed by sonication (Sonic Dismembrator 500, Fisher) in 1 mL cold PBS + internal standard. All samples were then worked up by solid phase extraction (Evolute Express ABN cartridge, 30 mg/1 mL, Biotage) using a Vacmaster manifold (Biotage). Columns were washed with dH₂O (1 mL), and analytes were eluted with MeOH (1 mL) and evaporated to dryness by high flow air purge. Samples were reconstituted in 200 μL 95:5 mobile phase A:B and analyzed by LC-MS/MS (2.0 μL injection volume). HPLC was performed using a Shimadzu Prominence HPLC (LCD-20AD) with temperature controlled autosampler (SIL-20AC). HPLC conditions: Phenomenex Kinetix[®] core column (2.6 μm, C18, 100 Å, 100 x 4.6 mm), mobile phase A = 25 mM ammonium formate in water (0.1% formic acid [FA]), mobile phase B = ACN (0.1% FA), 1.0 mL/min, 5% B to 95% B over 5 min followed by 95% B

for 2 min then 5% B for 5 min re-equilibration (total run time = 12 min). All samples were run in duplicate, and AUC for **5** and **6** were normalized to the intra-run IBA AUC to account for loss during workup. **5** concentrations were determined using std curve, while **6** was expressed as a percentage of **5** AUC.

Chapter 3

In vivo Ischemic Stroke Model: 6 Forms Preferentially in the Penumbra and Ischemic Lesion

Introduction

After the nitrone NXY-059 was found to significantly reduce infarct volume in permanent ischemia in pre-clinical studies,¹³⁰ it was advanced to clinical trials. Clinical administration of NXY-059 for the treatment of acute ischemic stroke used doses as high as 2270 mg over the first hour followed by 960 mg/h for three days (constant concentration 260 $\mu\text{mol/L}$) and showed no adverse effects compared to placebo.^{131,132} Drug was administrated within the first six hours of ischemia, and roughly a quarter of patients also received tissue plasminogen activator (TPA, Alteplase). NXY-059 was also clinically investigated for efficacy in treating intracerebral hemorrhage.¹³³ While NXY-059 appeared to be effective in the phase-II SAINT-I trial for ischemic stroke, it was deemed ineffective after the phase-III SAINT-II trial failed to observe evidence of efficacy for the primary (modified Rankin scale) or secondary outcomes (neurologic and activities-of-daily-living scales) at the six-hour time point,²⁸ and in a pooled analysis of both trials.¹³⁴ These results led to critical reflections on the hypothesis of neuroprotection for the treatment of stroke in humans,¹³⁵ however more recent trials suggest neuroprotection is possible, though initial treatment must occur in a shorter therapeutic window.¹³⁶⁻¹³⁸ Additionally, the pre-clinical models of stroke used to assess drug

candidates were determined to be critical – both permanent and transient animal models with heterogeneous populations are necessary to determine drug efficacy in later development phases, while early phases should focus on simpler stroke models to explore disease mechanisms and therapeutic targets.¹³⁹ With the clinical disappointment of NXY-059, newer nitrones were designed with the aim to improve upon its pre-clinical results and more thoroughly describe its mechanisms of action.

In addition to their ROS-trapping action, PBN-type nitrones were found to reduce tau formation in oligodendrocytes¹⁴⁰ and penumbra depolarizations, which are due to the release of K^+ and glutamate from the infarct core, after MCAO despite not reducing total infarct volume at the selected doses.^{141,142} The less-lipophilic, blood-brain barrier impermeable S-PBN improved neurological performance in a model of ischemic stroke, despite also giving mixed results in the measurement of infarct volume.^{143,144} Recent advances have led to increased potency, for example TBN is efficacious in both transient and permanent MCAO models of ischemic stroke, and was found to neutralize ROS and decrease Ca^{2+} influx.¹⁴⁵ An 80 mg/kg dose was most effective when given 2 hr after ischemia with decreasing efficacy observed at longer time points, epitomizing the previously mentioned recommendations for testing pre-clinical antioxidants. Furthermore, the potent nitron STAZN was found to be efficacious only at a ‘medium’ dose (0.7 mg/kg), whereas doses a factor of 10 lower or 50% higher showed no improvement.¹⁴⁶ Multiple initial dose windows were tested, from two to five hours post-ischemia, and it was found that a dosing regimen starting 2-3 hours after MCAO then twice daily significantly improved neurological function and infarct volume. The more recently developed cyclic nitrones have also been investigated for the treatment of *in vivo*

oxidative stress, and, when compared to linear nitrones like PBN, exhibit a longer half-life of the radical adduct, yet do not increase their efficacy.⁴¹ Such an increase in adduct stability is not desired for the design of the molecules herein, where their decomposition after ROS reaction is desired. Due to the exceptional safety profile of nitrones, and the clinical failure of NXY-059 and other compounds acting solely through antioxidant mechanisms, combination therapies with nitrones are currently being considered for stroke and other neurodegenerative diseases.¹⁴⁷⁻¹⁴⁹ The combination of NOS inhibitors and nitrones however, has seen little exploration.

NOS inhibition has not been clinically investigated for ischemic stroke, however the endogenously produced NOS inhibitors asymmetric / symmetric dimethyl arginine (ADMA/SDMA) are found to be increased in ischemic stroke, and may potentially be clinical biomarkers for worse stroke outcome.^{150,151} Despite higher levels correlating with higher mortality, the roles of ADMA/SDMA in ischemia are not yet fully understood, but it was observed that ADMA decreases cerebral blood flow and increases arterial stiffness which may contribute to cerebrovascular pathogenesis.¹⁵² As discussed in the previous chapters, exogenous NOS inhibitors have yielded conflicting pre-clinical results for the treatment of cerebral ischemic strokes,¹⁸ thus their clinical evaluation for stroke has not been undertaken. A nebulized iNOS inhibitor was used to investigate role of iNOS in COPD and asthma patients,^{153,154} and both selective eNOS and nNOS inhibitors were used to determine the extent of neutrally mediated vasoconstriction of hyperemia in patients administered adenosine.^{155,156} Concerns regarding the vasoconstriction potential of systemic NOS inhibitors is warranted. Under hypoxia, both NO and prostaglandins (PGs) are responsible for compensatory vasodilation, however it was observed that

inhibiting NOS alone did not eliminate this response in humans as vessels were able to dilate similar to normal vessels.^{157,158} This indicates that NOS inhibitors may not significantly inhibit the vasodilatory response of hypoxic tissue if inflammation can be prevented or neutralized. The non-selective NOS inhibitor L-*N*(γ)-monomethylarginine (L-NMMA, Tilarginine) was clinically tested for the treatment of acute myocardial infarction (MI), characterized by a blockage of coronary arteries resulting in ischemia, for its effect on arterial pressure.^{159,160} Similar to ischemic stroke, MI results in a state of oxidative stress exacerbated at least in part by uncoupled NOS,¹⁶¹ however a compensatory increase in functional NOS activity can lead to cardiogenic shock.¹⁶² L-NMMA was found to provide no significant benefit over placebo for the treatment of MI and cardiogenic shock despite promising pre-clinical and small-scale clinical data.^{163,164} This was hypothesized to be due to a lack of NOS isoform selectivity and systemic inhibition, each of which would cause undesirable off-target effects such as peripheral vasoconstriction that may cancel the desired beneficial effects at the site of ischemia.¹⁶⁵

The clinical paths of L-NMMA and NXY-059 are remarkably similar, and highlight the same problems in the treatment of ischemia with antioxidants and NOS inhibitors: Too much focus on a singular mechanism (e.g. anti-oxidation alone) and a lack of ischemia site-specificity. This study aims to overcome these shortcomings with a ROS-sensitive NOS inhibitor, **5**. As described above, **5** is able to neutralize ROS to then decompose to the NOS inhibitor **6** preferentially in ischemic tissue to provide region selectivity and significantly improved neuroprotection and anti-inflammation over conventional nitrones. For these reasons, the effects of **5** and the decomposition product **6** were explored in a mouse model of permanent ischemia.

Results

Effect of **5** on Cerebral Blood Flow (CBF)

Due to the complex interplay of $O_2^{\cdot -}$ -scavenging, NO donation, and NOS inhibition, the acute effects of **5** on CBF were investigated. CBF correction in acute ischemic stroke has a strong correlation with positive patient outcome, and is an often overlooked aspect of neuroprotective therapies.¹⁶⁶ Doppler flowmetry measurement over the area of the middle cerebral artery (MCA) for 20 min after intravenous administration was performed on anesthetized, C57BL/6 mice. Doppler flowmetry measures the relative Doppler shift of a laser positioned over a blood vessel caused by the movement of vascular red blood cells. A known NO donor and vasodilator, isoamyl nitrate (20 mg/kg), was found to increase CBF over 20 min ($111\% \pm 4.0\%$ baseline, $p < 0.05$). Paradoxically, a lower dose of **5** (1 mg/kg in sterile normal saline) caused a significant increase in CBF ($126\% \pm 4.6\%$ baseline, $p < 0.001$), while a higher dose (10 mg/kg) produced baseline readings ($102\% \pm 0.7\%$ baseline, **Figure 3-1**). Compared to isoamyl nitrate, the low dose of **5** increased CBF at an earlier time point (20 min and 5 min, respectively). Plasma and brain samples collected immediately after sacrifice (25 min from drug administration) and analyzed by LC-MS/MS confirmed that the animals in the higher-dose group were exposed to larger doses of **5** in plasma ($1,681 \pm 881$ nM for 10 mg/kg; 171.7 ± 51.8 nM for 1 mg/kg) and brain (17.97 ± 2.23 nM for 10 mg/kg; 6.41 ± 2.03 nM for 1 mg/kg). A similar effect was previously observed by Inanami and Kuwabara¹⁶⁷ where administration of the NOS-inhibitor L-NAME abrogated an increase in MCA blood flow after PBN administration in rats.

The average brain:plasma ratio after 25 min for the two treatments of **5** was found to be quite poor (0.029 ± 0.010 , $n = 6$) which may be explained by the positively-charged amidine moiety at physiological pH akin to that of the negatively-charged S-PBN and NXY-059. The decomposition product **6** was detected by LC-MS/MS using the MRM method attained previously, and was observed to be present at higher levels in the brain than plasma ($24.5\% \pm 0.1\%$ and $12.7\% \pm 0.2\%$, respectively, expressed as percent of **5** AUC within the same run, $n = 6$). This difference was not found to be significant by an unpaired t-test ($p = 0.18$), but may reach significance with a larger number of animals. Additionally, more data points are required to elucidate the dose-response correlation and the time to return to baseline CBF after **5** administration.

Figure 3-1. Doppler CBF measurement after intravenous injection of isoamyl nitrate or **5** to anesthetized mice over 20 minutes relative to baseline. The lower dose of **5** (1 mg/kg) elevated CBF, whereas a higher dose of **5** (10 mg/kg) showed no change in CBF from baseline (n = 3, One-way ANOVA followed by Newman-Keuls *post hoc* test). Data represented as mean $\pm$ SEM, *p < 0.05, ***p < 0.001 from baseline.

***In vivo* Neuroprotection in a Murine Model of Permanent Ischemia**

After exploring the effects of chosen doses of **5** on CBF, the neuroprotective effects of **5** were explored in a whole animal model of permanent ischemia. Wild-type, male C57BL/6 mice were subjected to permanent distal MCA occlusion (pMCAO) followed by a bi-phasic dosing regimen of **5** to assess *in vivo* neuroprotection and effects on neurobehavioral parameters. Vehicle (n = 10) or **5** (10 mg/kg in normal saline, n = 12) were administered intravenously by lateral tail vein injection at 3 and 6 hr after pMCAO followed by 1 mg/kg **5** twice daily separated by 8 hr thereafter. Both doses were well tolerated, and there were no observed adverse effects at any time point. Animals were trained on neurobehavioral assessments for 3 days prior to surgery, then were tested at 24, 48 and 72 hr post-ischemia.

No effect on rotarod performance, which measures the animals' latency to fall from a rotating rod, was observed for both treatment groups, which remained near baseline levels after ischemia (**Figure 3-2**). **5** treatment trended toward an increase in rotarod performance at 72 hr from vehicle, however this was not statistically significant. Vehicle treated animals showed a decline in forelimb grip strength after pMCAO, while the **5**-treated group did not exhibit a post-surgical decline, and obtained higher readings than vehicle treated animals at each time point. Neurological deficit scoring (NDS), a 28-point system optimized by our laboratory, was conducted prior to sacrifice at 72 hr, in which **5**-treated animals showed a less-severe deficit than vehicle treated animals (4.2 ± 0.4 and 5.3 ± 0.4 , respectively, $p < 0.05$, unpaired t-test). **5** treatment appeared to moderately ameliorate neurobehavioral deficits induced by permanent ischemia while showing good tolerability and no adverse effects for acute administration.

Figure 3-2. Neurobehavioral performance of mice treated with vehicle (n = 10) or **5** (n = 12) after pMCAO relative to pre-ischemia baseline. No significant differences were observed for rotarod performance (**A**), but treatment with **5** improved grip strength (**B**, Two-way ANOVA followed by Bonferroni multiple comparisons test), and reduced neurological deficit scores (**C**, unpaired t-test). Data represented as mean $\pm$ SEM, *p < 0.05, **p < 0.01, ***p < 0.001 from vehicle or baseline.

Animals from both groups were sacrificed by CO₂ 72 hr post-pMCAO (4 hr from the last dose), brains were rapidly harvested, sectioned into 2-mm thick coronal slices, and stained by 2,3,5-Triphenyl-tetrazolium chloride (TTC) in normal saline to visualize the infarct area (**Figure 3-3**). TTC stains viable tissue red, while leaving the dead tissue of the ischemic core and penumbra white. Sections were quickly imaged, and infarct volume was determined by the ratio of unstained tissue in the ipsilateral cortex to the total contralateral area using a method described previously for estimating neuroprotection of NXY-059 in permanent ischemia.^{130,168} Mice treated with **5** were found to have a 37% decrease in infarct volume compared to vehicle animals ($p < 0.05$, unpaired t-test). In **5**-treated mice, the dead tissue in the penumbra was observed to be reduced in volume compared to vehicle controls despite a similar ischemic core. It is hypothesized that, analogous to the conversion of **5** to **6** in OGD-treated cells *in vitro*, **5** would be decomposed in the penumbra and ischemic areas in larger amounts than in the contralateral areas or periphery resulting in the observed efficacy.

Figure 3-3. Infarct volume analysis (**A**) and representative TTC-stained sections (**B**) of vehicle (top) vs **5**-treated (bottom) mouse brains 72 hr after pMCAO by TTC staining. Treatment with **5** significantly reduced the infarct volume induced by pMCAO. Data represented as mean $\pm$ SEM, unpaired t-test, * $p < 0.05$.

LC-MS/MS Analysis of Stroke Brain Regions after 5 Treatment

Directly after imaging (within 1 hr), sections of **5**-treated brain regions were rapidly frozen at -80 °C pertaining to ischemic (no TTC staining), penumbra (mild staining), and contralateral (full staining) areas. These sections were chosen as representative of a full spectrum of ischemic damage after pMCAO. Samples were worked up as with *in vitro* OGD samples above to assess the levels of **5** and **6** formation. Preliminary LC-MS/MS analysis of these regions for the levels of **5:6** show decreasing levels of **6** and increasing levels of **5** from the core of the ischemic area (high oxidative stress) outward towards the contralateral area (reduced oxidative stress, **Figure 3-4**). This data supports the hypothesis that **5** would be converted to **6** at higher levels in tissues undergoing oxidative stress, and confirms the observations of higher **6** formation in OGD-treated cells *in vitro*. This conversion can be used to identify not only NOS inhibitor formation, but tissue-ROS formation and nitron-NO formation. A larger number of samples are required for statistical analysis of the tissue-selectivity, but the preliminary data here coupled with the *in vitro* data and literature precedent strongly support this hypothesis.

Figure 3-4. Preliminary LC-MS/MS analysis of **5** and **6** levels in ischemic (A) penumbra (B) and contralateral (C) brain regions of mice 72 hr after pMCAO. Brains were rapidly removed after sacrifice by CO₂, sectioned into 2 mm coronal slices, and stained by TTC to visualize the ischemic area. **6** was observed to be formed at higher levels in ischemic tissues, giving way to higher levels of the parent compound, **5**, moving outward from the infarct area.

Discussion

5 has a Dose-Dependent Effect on CBF

The strongest evidence of NOS inhibition by **5** and/or **6** was observed through the measurement of CBF by Laser Doppler Flowmetry over the MCA region of treated mice. Intravenous injection of 10 mg/kg **5** in normal saline to anesthetized mice gave no change in CBF from baseline, yet a lower dose, 1 mg/kg, caused a significant increase in CBF over 20 min. When looking for literature precedent, a study by Inanami and Kuwabara¹⁶⁷ showed a dose-dependent increase of MCA CBF in rats with PBN alone, which was lowered to baseline upon co-administration with L-NAME. The CBF increasing effects of **5** are likely due to a combination of scavenging vasoconstrictive $O_2^{\cdot -}$, and the releasing of vasodilatory NO, allowing a larger effect than the NO-donor isoamyl nitrate. Acute NOS-inhibitor exposure is observed to cause hypertension and bradycardia at doses as low as 3 mg/kg of L-NAME.¹⁶⁹ It is unclear if the CBF lowering effects herein are due to excess **5**, given its predicted affinity for NOS, or the formation of **6**, which was detected in plasma by LC-MS/MS. The *in vitro* Griess assay of activated microglia in the previous chapter suggests that **5** does not have iNOS inhibition properties, and the predicted binding of **5** with eNOS in the first chapter is less than that of iNOS, thus it is unlikely to inhibit vascular eNOS at the administered doses. This observation could be confirmed by supplementing a low dose of **6** to the low dose of **5**, or administering **6** alone and measuring CBF. It is predicted that in each case **6** will lower CBF due to its confirmed NOS inhibition properties. Levels of **6** were higher than expected in **5**-treated animals after only 25 min exposure, which indicates a short half-life of **5**, a common concern for antioxidants and nitrones in particular. For this reason, a higher loading dose of **5** was

chosen for the pMCAO study, followed by a lower dose twice daily – a regimen that has found success for experimental nitrones in ischemia as discussed previously.¹⁴⁶ Also, the level of **6** formation at 25 min is without insufficient to draw conclusions about stability. A full pharmacokinetic study in healthy mice and those undergoing oxidative pathology (e.g. stroke) would allow the study of the rate of **5** decomposition and **6** formation *in vivo* to provide the full picture of metabolic stability, and oxidative stress specificity. The blood flow increase monitored herein was only that of CBF, which is vital in the context of acute ischemia, yet the full effect of **5** and **6** on peripheral blood flow was not measured, and would be relevant in populations with cardiovascular co-morbidities common to stroke. Inanami and Kuwabara observed a reduction in peripheral blood pressure with PBN that was prevented with L-NAME, corresponding to the CBF measurements. It is expected that **5** will show similar effects on blood pressure as with CBF. Future studies will look to examine the full effect of this novel class of molecules on the cardiovascular system beyond that of the cerebrovascular environment, and optimize the dose administered for the desired effect on CBF in ischemia.

6 Forms Preferentially in the Penumbra and Ischemic Lesion

Animals treated with **5** exhibited a > 30% reduction in infarct volume 72 hr after pMCAO as measured by TTC, coupled with significant improvements in neurobehavioral assay performance. The dose and regimen chosen mirror that of previously assayed nitrones,¹⁴⁶ and follows the recent guidelines for the development of neuroprotective agents in ischemic stroke.¹³⁶⁻¹³⁸ The concept of bolus loading doses of **5** were taken from the SAINT-I and II trials for NXY-059, and chosen with the aim to provide high levels of antioxidant to the ischemic area at the onset of ischemia. The reduction in pMCAO-

induced infarct volume agrees with the previously described neuroprotection in the *in vitro* OGD assay. Western blotting of OGD-treated neuronal cultures suggests this protection to be due to Akt activation, and the decrease of 3-NT formation and Caspase-3 cleavage. Correction of the ROS/NO imbalance by **5** & **6** after ischemia and reperfusion is suggested to provide the neuroprotection observed *in vitro* and *in vivo*.

The translation of *in vitro*, OGD-induced formation of **6** to *in vivo* ischemia was investigated by LC-MS/MS. Analysis of ischemic, penumbra, and contralateral sections of mouse brains exposed to pMCAO and **5** treatment show increasing formation of **6** from the contralateral to ischemic areas – in the direction of increasing oxidative stress (**Figure 3-4**). Parallel to the *in vitro* and ROS experiments, the NOS inhibitor **6** is formed *in vivo* preferentially under conditions of oxidative stress, and here preferentially to the pathologic area. NXY-059 was found to increase pAkt/Akt levels in brains of animals after transient MCAO specific to the infarct and penumbra areas.¹¹⁷ While the authors attributed this to cell surface interactions, it is more likely to be due to increased reactivity of the nitron in those areas, leading to ROS neutralization and NO release, and thus Akt activation. This hypothesis is supported by the data herein which shows greater decomposition of **5** under oxidative conditions *in vitro* and *in vivo* analogous to that of the ROS chemical systems. Utilizing this site-specific property can unlock new avenues of drug targeting in ischemia and other various pathologies characterized by oxidative stress.

The oxidative stress induced during the onset of ischemia is only one phase of neuronal death during stroke, albeit a major one. The secondary injury to neurons occurs upon the activation of microglia which then produce damaging cytokines and excessive

NO.^{170,171} Targeting microglial activation after stroke has been recently investigated and was found to significantly suppress the evolution of ischemic damage at later time points (e.g. 24 to 48 hr).¹⁷²⁻¹⁷⁴ Similarly, astrocytes are activated after ischemia/reperfusion, and also increase iNOS expression in the days following ischemia.^{175,176} The iNOS inhibitor 1400W was found to be neuroprotective in an animal model of transient ischemia,¹⁷⁷ and co-treatment with an NO-donor increased functional recovery of perfused skeletal muscle.¹⁷⁸ In addition to targeting the uncoupled NOS induced by ischemia, **5** treatment, through **6** formation, is likely able to reduce iNOS activity through its inhibition as observed in the Griess assay of activated microglia. Thus, the initial bolus of **5** would provide neuroprotection from oxidative stress, as is typical of classical antioxidants, and the formation of **6** would prevent secondary inflammatory injury caused by iNOS expressed by activated microglia and astrocytes after ischemia. Future studies will explore the effects of **5** treatment on oxidative stress, both in the CNS and periphery, and the levels of glial activation at the site of ischemia.

Future Directions – Nitrones as CNS-Permeable, ROS-Sensitive Prodrugs

Despite being charged at physiological pH, **5** and **6** were both detected in the CNS by LC-MS/MS of brain tissue. Future analogues will aim to increase permeability while fighting the prototypical hydrophilicity of NOS-inhibitors and increasing isoform specificity. The ideal NOS-inhibitor for ischemia will possess dual iNOS/nNOS inhibition for the inhibitor and not the nitrone pro-drug with fold-increased selectivity against eNOS, and higher lipophilicity to allow blood-brain barrier penetration. Future syntheses will take advantage of the one-step synthesis of nitrones from their corresponding aldehydes.³⁸ The advantages of this method are two-fold: ease of synthesis

to allow derivatization, and, more importantly, it will allow the aldehyde NOS inhibitors to be characterized and screened prior to formation of their corresponding nitron. The molecule described here, **5**, demonstrates the potential of nitrones to act as more than antioxidants, but as carrier molecules that are able to provide an additional, site-specific action. NOS was explored as one such target due to its unique dual-roles under oxidative pathology, however other molecular pathways could be targeted using the above methodologies. The full effect of **5** and **6** on each NOS isoform should be elucidated in future works, and their effects on the full cardiovascular system are essential due to the potential peripheral effects of NOS inhibition. Overall this study outlines the direction of the future development of nitron-NOS inhibitors for the site-specific inhibition of NOS under conditions of oxidative stress: The screening of putative NOS inhibitors for isoform selectivity and anti-inflammatory activity, nitron-NOS inhibitor synthesis and characterization, testing the ability of NOS inhibitors to be formed from their respective nitrones after reacting with ROS, and lastly assessing the neuroprotective potential of the nitron-NOS inhibitor *in vitro* and *in vivo*.

Experimental

Animals

All animal protocols were approved by the University of Toledo Health Science Campus Institutional Animal Care and Utilization Committee, and all NIH guidelines were followed. Male C57BL/6 mice, 6-8 weeks old at 23-25 g (Charles River) were housed with a 12 hr light/dark cycle at 22 ± 1 °C.

Permanent Middle Cerebral Artery Occlusion (pMCAO)

The distal part of the MCA was permanently occluded as previously optimized by our laboratory.^{73,179} Briefly, mice were anesthetized with 1% isoflurane, the site was aseptically cleaned, and a small 1.0 cm incision was made between the left eye and ear. The temporal muscle was moved aside to view the MCA under the temporal bone, and a 2 mm hole was drilled with a dental drill directly over the distal MCA. The artery was directly occluded with a bipolar coagulator, and the incision was sutured. The animals' body temperature was maintained at 37.0 ± 5 °C throughout the procedure and after surgery until recovery. Animals were intravenously injected by lateral tail vein with 100 μ L normal saline (n = 10) or 10 mg/kg **5** in vehicle (n = 12) at 3 and 6 hr after pMCAO. Either vehicle or 1 mg/kg **5** were then dosed twice daily thereafter, spaced 8 hr apart, until sacrifice by CO₂ inhalation at 72 hr post-pMCAO.

Laser Doppler Cerebral Blood Flow (CBF)

Animals were anesthetized and the incision was performed as above to expose the temporal bone, through which the MCA was visible. The end of a fiber optic cable fitted to a MoorVMS-LDF Laser Doppler Monitor (Moor Instruments) was positioned in place over the MCA. The baseline CBF as flux was monitored for 5 min prior to intravenous injection by lateral tail vein of normal saline vehicle, isoamyl nitrate (20 mg/kg), or **5**. Measurements were taken every 5 minutes in triplicate for each animal, and reported as ratio to baseline. Animals were sacrificed at 25 min by CO₂ inhalation, blood was collected into K₂EDTA coated tubes (BD Microtainer®), and brains were rapidly dissected out and frozen on dry ice for LC-MS/MS analysis.

Neurobehavioral Assays

Mice were trained on neurobehavioral paradigms for 3 days prior to pMCAO, and baseline readings were recorded 24 hr prior to surgery. For rotarod analysis, mice were placed on a moving rod (Columbus Instruments) programmed to rotate at 1 rpm and accelerate by 1 rpm every 10 sec until the animal falls from the rod. The latency to fall was recorded manually and reported as ratio to baseline. Grip strength analysis was performed using a grip strength meter (Columbus Instruments) fitted with a pull bar assembly. Forelimbs of mice were placed on the bar and peak force until release when pulled by the tail was displayed on the digital display and manually noted. Neurological deficit scoring (NDS) was conducted 72 hr after pMCAO prior to sacrifice, and evaluated on a 28-point score pattern optimized by our laboratory.^{73,180} The total score was determined by the sum of the seven criteria graded from 0 to 4, with higher scores indicating more severe deficits.

Infarct Volume

Brains of animals sacrificed by CO₂ inhalation 72 hr after pMCAO were rapidly dissected out, sliced into five 2 mm-thick coronal sections, and incubated in warm 1% triphenyltetrazolium chloride (TTC, Sigma) in normal saline. Infarct volumes were estimated as described previously for NXY-059,^{130,168} briefly by measuring rostral and caudal sides of each section in conjunction with the thickness, and expressed as a percentage of the volume of the contralateral hemisphere. After imaging, portions of the ischemic lesion, penumbra, and contralateral tissue were quickly frozen on dry ice for LC-MS/MS analysis.

LC-MS/MS

Synthesized **5**, and purchased 4(*1H*-Imidazol-1-yl)benzaldehyde (IBA, Sigma) were used to determine LC-MS/MS fragmentation. Isolated **6** from the **5**-ONOO⁻/HCl system was used for **6** fragmentation. MRM analysis of IBA (*m/z* 173.10 → 91.10, 104.10, 117.05; RT: 4.13 min), **5** (*m/z* 248.20 → 148.00, 134.15, 190.05; RT: 4.81 min), and **6** (*m/z* 177.00 → 119.10, 90.95, 65.15; RT: 3.87 min) were determined. A standard curve for **5** was generated using pure samples applied to blank matrix (0.01 μM, 0.1 μM, 0.5 μM, 1.0 μM, and 5.0 μM; $R^2 = 0.99$).

Blood samples (100 μL) were spiked with internal standard, and centrifuged to collect the supernatant. Brain samples were weighed, homogenized by a Bullet Blender Storm (Next Advance Inc.) in 500 μL cold PBS + internal standard for 1 min at 4 °C, then centrifuged to collect the supernatant. All samples were then worked up by solid phase extraction (Evolute Express ABN cartridge, 30 mg/1 mL, Biotage) using a Vacmaster manifold (Biotage). Columns were washed with dH₂O (1 mL), and analytes were eluted with MeOH (1 mL) and evaporated to dryness by high flow air purge. Samples were reconstituted in 200 μL 95:5 mobile phase A:B and analyzed by LC-MS/MS (2.0 μL injection volume). All samples were run in duplicate, and AUC for **5** and **6** were normalized to the intra-run IBA AUC to account for loss during workup. **5** concentrations were determined using std. curve, while **6** was expressed as a percentage of **5** AUC.

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Appendix A

Supporting Information

^1H , ^{13}C NMR, HPLD-PDA purity, and HRMS for synthesized compounds, and computational data for *in silico* experiments.

Figure A-1. NMR/HPLC/LRMS of 2

Figure A-2. NMR/HPLC/LRMS of 3

Figure A-3. NMR/HPLC/LRMS of 4

Figure A-4. NMR/HPLC/LRMS of **5**

Compound	PBN	PBN-O2	PBN-O2H	PBN-OH	PBN-COONO	PBN-COONO Triplet	PBN-tHOONO Triplet
file	PBN.log	PBN-O2.log	PBN-O2H.log	PBN-OH.log	PBN-COONO.log	PBN-COONO-DR	PBN-tHOONO-b.log
E-6-3t+g* tight	-558.1753974	-708.6295898	-709.1215789	-633.9888874	-838.6426924	-838.5387083	-839.0780859
E-6-3t+g*	-558.1229151	-708.4689312	-709.05267	-633.9248441	-838.485894	-838.3831309	-839.0079195
ZPE (raw)	0.2402741	0.2452808	0.2582605	0.2553993	0.2493463	0.2521946	0.265531
Therm Corr to H (raw)	0.254034	0.260894	0.274992	0.270696	0.270432	0.270965	0.285543
Therm Corr to G (raw)	0.199693	0.203307	0.214213	0.213551	0.196311	0.204959	0.212382
H (raw)							
G (raw)							
ZPE (scaled)	0.235613	0.240522	0.253250	0.250445	0.244509	0.247302	0.260380
Electronic State	1-A	2-A	2-A	2-A	1-A	3-A	3-A
s2-6-3t+g*	0	0.754042	0.753684	0.753597	0	2.009709	2.007412
Symmetry	-	-	-	-	-	-	-
N lmg	0	0	0		0	0	0
Rotational Con. (GHz)	2.0228295	1.1568872	1.0626812	1.5260768	0.6130537	0.6496567	0.5317328
Rotational Con. (GHz)	0.4019737	0.3895863	0.3800827	0.3833158	0.2675786	0.3560352	0.330638
Rotational Con. (GHz)	0.3626709	0.3302713	0.3457177	0.3771236	0.2542705	0.2596329	0.250924
H298		7.3	-10.2	-43.7	-44.8	20.8	-31.0
6-3t+g* tight	-557.926025	-708.373454	-708.851597	-633.723146	-838.377098	-838.272636	-838.797694
6-3t+g* tight	-557.873542	-708.212796	-708.782688	-633.659103	-838.220299	-838.117058	-838.727528
G298		19.8	2.1	-32.7	-38.1	32.5	-23.4
6-3t+g* tight	-557.980366	-708.431041	-708.912376	-633.780291	-838.451219	-838.338642	-838.870855
6-3t+g* tight	-557.927883	-708.270383	-708.843467	-633.716248	-838.294420	-838.183064	-838.800689

Compound	Nitrones5	Nitrones5-O2	Nitrones5-O2H	Nitrones5H	Nitrones5H-O2	Nitrones5H-O2H	6
file	Nitrones5.log	Nitrones5-O2.log	Nitrones5-O2H.log	Nitrones5H.log	Nitrones5H-O2.log	Nitrones5H-O2H	Inh5H-Ald.log
E-6-31+g** tight	-785.6357585	-936.0904947	-936.5851853	-786.1034327	-936.5828289	-937.0470166	-573.5323419
E-6-31g*	-785.553248	-935.902876	-936.4902325	-785.9601173	-936.4881889	-936.8975349	-573.4013846
ZPE (raw)	0.3364848	0.3413772	0.3551733	0.3496388	0.3536872	0.3677559	0.2193848
Therm Corr to H (raw)	0.356426	0.36327	0.377663	0.37001	0.376723	0.391074	0.233299
Therm Corr to G (raw)	0.286235	0.289719	0.301437	0.299859	0.299649	0.313683	0.178218
H (raw)							
G (raw)							
ZPE (scaled)	0.329957	0.334754	0.348283	0.342856	0.346826	0.360621	0.215129
Electronic State	1-A	2-A	2-A	1-A	2-A	2-A	1-A
s2-6-31g*	0	0.750012	0.754036	0	0.753653	0.753838	0
Symmetry	-	-	-	-	-	-	-
NImag	0	0	0	0	0	0	0
Rotational Con. (GHz)	0.6810234	0.8199599	0.5214058	0.9140464	0.49675	0.447046	1.7985325
Rotational Con. (GHz)	0.1725886	0.1293767	0.1597197	0.1448533	0.16514	0.2003035	0.3241078
Rotational Con. (GHz)	0.1465231	0.1226153	0.1520779	0.1348986	0.14509	0.1661309	0.301647
H298		6.9	-12.1		-8.6	-8.5	
6-31+g** tight	-785.285860	-935.733847	-936.214413	-785.740206	-936.212967	-936.663077	-573.303299
6-31g* tight	-785.203350	-935.546229	-936.119460	-785.596890	-936.118327	-936.513595	-573.172342
G298		19.3	0.4		1.6	3.2	
6-31+g** tight	-785.356051	-935.807398	-936.290639	-785.810357	-936.290041	-936.740468	-573.358380
6-31g* tight	-785.273541	-935.619780	-936.195686	-785.667041	-936.195401	-936.590986	-573.227423

Compound	<u>NSH-cOONO-Triplet</u>	<u>NSH-tOONO-Triplet</u>	<u>NSH-CHOONO-Triplet</u>	<u>NSH-tHOONO-Triplet</u>
file	Nitrone5H-cOONOr.c	Nitrone5H-tOONOr.com	Nitrone5H-CHOONOr.cc	Nitrone5H-tHOONOr.c
E-6-31+g** tight	-1066.526598	-1066.465825	-1067.005068	-1067.003533
E-6-31g*	-1066.41267	-1066.351577	-1066.846266	-1066.849762
ZPE (raw)	0.3604898	0.3621789	0.3751124	0.3752402
Therm Corr to H (raw)	0.385945	0.386455	0.401823	0.401728
Therm Corr to G (raw)	0.302557	0.308231	0.311996	0.314722
H (raw)				
G (raw)				
ZPE (scale d)	0.353496	0.355153	0.367835	0.367961
Electronic State	3-A	3-A	3-A	3-A
s 2-6-31g*	2.007249	2.008104	2.006967	2.007148
Symmetry	-	-	-	-
NImag	0	0	0	0
Rotational Con. (GHz)	0.45994	0.4844277	0.3927496	0.4906829
Rotational Con. (GHz)	0.1585	0.1642448	0.1569542	0.1437159
Rotational Con. (GHz)	0.1291	0.133341	0.1308911	0.1244843
H298	-17.4	16.8	-27.0	-29.3
6-31+g** tight	-1066.147647	-1066.086396	-1066.610522	-1066.609084
6-31g* tight	-1066.033718	-1065.972148	-1066.451721	-1066.455314
G298	-6.7	30.9	-19.9	-20.4
6-31+g** tight	-1066.231035	-1066.164620	-1066.700349	-1066.696090
6-31g* tight	-1066.117106	-1066.050372	-1066.541548	-1066.542320

Compound	NSH-coONO	NSH-tONO	NSH-chONO	NSH-tHONO	
file	Nitrone5H-coON	Nitrone5H-tON	Nitrone5H-chOO	Nitrone5H-tHONO.	
E-6-31+g** tight	-1066.593684	-1066.581989	-1067.005376	-1067.006791	
E-6-31g*	-1066.48573	-1066.477735	-1066.864457	-1066.862848	
ZPE (raw)	0.3590003	0.3587707	0.3780774	0.377909	
Therm Corr to H (ra	0.385789	0.386051	0.402402	0.402327	
Therm Corr to G (ra	0.299769	0.294497	0.325924	0.325611	
H (raw)					
G (raw)					
ZPE (scaled)	0.352036	0.351811	0.370743	0.370578	
Electronic State	1-A	1-A	1-A	1-A	
s2-6-31g*	0	0	0	0	
Symmetry	-	-	-	-	
Nl mag	0	0	0	0	
Rotational Con. (GHz	0.3247771	0.2938049	0.477344	0.4580586	
Rotational Con. (GHz	0.209315	0.1094194	0.1921315	0.1941952	
Rotational Con. (GHz	0.1535112	0.0904139	0.1627655	0.1616104	
H298	-59.6	-56.3	-26.9	-31.0	
6-31+g** tight	-1066.214860	-1066.202898	-1066.610309	-1066.611795	
6-31g* tight	-1066.106905	-1066.098644	-1066.469389	-1066.467852	
G298	-50.5	-50.6	-11.4	-15.6	
6-31+g** tight	-1066.300880	-1066.294452	-1066.666787	-1066.688511	
6-31g* tight	-1066.192925	-1066.190198	-1066.545867	-1066.544568	

NSH-O2 + 2H2O	Nitrone5H-OH	
Nitrone5H-O2-H2	Nitrone5H-OH.106	
-1089.490779	-861.9135897	
-1089.360431	-861.7677685	
0.406076	0.3648808	
0.433394	0.386758	
0.347854	0.312947	
0.398198	0.357802	
2-A	2-A	
0.753687	0.753771	
-	-	
0	0	
0.4628079	0.5085957	
0.1314551	0.2251992	
0.1193807	0.1785217	
-19.2	-41.5	
-1089.065263	-861.533910	
-1088.934915	-861.388089	
12.6	-31.1	
-1089.150803	-861.607721	
-1089.020455	-861.461900	

Compound
file	<u>HO₂</u>	<u>O₂</u>	<u>HO</u>	<u>H₂O</u>	<u>COONO</u>	<u>tOONO</u>	<u>CHOONO</u>	<u>tHOONO</u>
superH.com.log	super.com.log							
E-6-31+g** tight	-150.926851	-150.465075	-75.7389891	-76.4422582	-280.3954815	-280.3883917	-280.8553735	-280.8492933
E-6-31g*	-150.8991565	-150.2996938	-75.7234548	-76.4089533	-280.2500382	-280.2434027	-280.8315481	-280.8212281
ZPE (raw)	0.014031	0.002761	0.0083041	0.0211685	0.0114255	0.0108351	0.0236257	0.0225121
Therm Corr to H (ra	0.017832	0.006082	0.011609	0.024947	0.015985	0.015605	0.028533	0.027479
Therm Corr to G (ra	-0.00816	-0.017041	-0.008638	0.003501	-0.014355	-0.01486	-0.002383	-0.00353
H (raw)								
G (raw)								
ZPE (scaled)	0.013759	0.002707	0.008143	0.020758	0.011204	0.010625	0.023167	0.022075
Electronic State	2-A''	-	1-A	1-A	1-A	1-A	1-A	1-A
s2 6-31g*	0.754	0.756	0.751967	0	0	0		0
Symmetry	-	-	-	-	-	-	-	-
NImag	0.000000	0.000000	0	0	0	0		0
Rotational Con. (Gh	608.8470994	0	0	787.909196	24.1507663	76.2586328	21.4709468	57.601434
Rotational Con. (Gh	33.5599447	34.5113404	551.744617	432.3087586	7.6620056	5.10126	7.956318	4.971635
Rotational Con. (Gh	31.8067418	34.5113404	551.744617	279.146894	5.8166359	4.7814114	5.8051498	4.5766224
H298								
6-31+g** tight	-150.909291	-150.459047	-75.727541	-76.417722	-280.379718	-280.372997	-280.827299	-280.822251
6-31g* tight	-150.881597	-150.293665	-75.712007	-76.384417	-280.234275	-280.228008	-280.803473	-280.794186
G298								
6-31+g** tight	-150.935283	-150.482170	-75.747788	-76.439168	-280.410058	-280.403462	-280.858215	-280.853260
6-31g* tight	-150.907589	-150.316788	-75.732254	-76.405863	-280.264615	-280.258473	-280.834389	-280.825195

Figure A-5. Thermodynamic parameters of ROS - PBN and **5** adducts at the PCM/B3LYP/6-31+g**//B3LYP/6-31g* level of theory (ZPE scaling factor = 0.9806 and hartree to kcal/mol = 627.5095).

<u>5-eNOS(L-Arginine)</u>		<u>5-nNOS(L-Arginine)</u>		<u>5-iNOS(L-Arginine)</u>	
Energy	-15.716	Energy	-16.558	Energy	-23.7129
SimpleFitness	-15.716	SimpleFitness	-16.558	SimpleFitness	-23.7129
FullFitness	-2026.81	FullFitness	-2187.41	FullFitness	-2238.81
InterFull	-100.245	InterFull	-89.6863	InterFull	-112.053
IntraFull	9.10902	IntraFull	1.8422	IntraFull	2.98932
solvFull	-2233.58	solvFull	-2412.77	solvFull	-2434.46
surfFull	297.909	surfFull	313.206	surfFull	304.712
extraFull	0	extraFull	0	extraFull	0
deltaGcompsolvpol	-2233.58	deltaGcompsolvpol	-2412.77	deltaGcompsolvpol	-2434.46
deltaGcompsolvnonpol	297.909	deltaGcompsolvnonpol	313.206	deltaGcompsolvnonpol	304.712
deltaGprotolvpol	-2268.57	deltaGprotolvpol	-2460.55	deltaGprotolvpol	-2486.33
deltaGprotolvnonpol	299.405	deltaGprotolvnonpol	315.706	deltaGprotolvnonpol	306.538
deltaGlgsolvpol	-35.9009	deltaGlgsolvpol	-34.6086	deltaGlgsolvpol	-34.9496
deltaGlgsolvnonpol	6.88548	deltaGlgsolvnonpol	6.5882	deltaGlgsolvnonpol	6.59309
deltaGvdw	-100.245	deltaGvdw	-89.6863	deltaGvdw	-112.053
deltaGelec	0	deltaGelec	0	deltaGelec	0
deltaG	-9.92754	deltaG	-8.29188	deltaG	-10.0733
Cluster	0	Cluster	8	Cluster	0
ClusterRank	1	ClusterRank	0	ClusterRank	0
<u>5-eNOS(1400W)</u>		<u>5-nNOS(1400W)</u>		<u>5-iNOS(1400W)</u>	
Energy	-14.9436	Energy	-23.1567	Energy	-26.572
SimpleFitness	-14.9436	SimpleFitness	-23.1567	SimpleFitness	-26.572
FullFitness	-3989.59	FullFitness	-2516.59	FullFitness	-4312.7
InterFull	-107.636	InterFull	-100.051	InterFull	-117.937
IntraFull	10.5244	IntraFull	2.66276	IntraFull	3.10626
solvFull	-4407.65	solvFull	-2732.51	solvFull	-4800.8
surfFull	515.174	surfFull	313.304	surfFull	602.93
extraFull	0	extraFull	0	extraFull	0
deltaGcompsolvpol	-4407.65	deltaGcompsolvpol	-2732.51	deltaGcompsolvpol	-4800.8
deltaGcompsolvnonpol	515.174	deltaGcompsolvnonpol	313.304	deltaGcompsolvnonpol	602.93
deltaGprotolvpol	-4451.84	deltaGprotolvpol	-2768.03	deltaGprotolvpol	-4850.39
deltaGprotolvnonpol	516.682	deltaGprotolvnonpol	314.623	deltaGprotolvnonpol	604.61
deltaGlgsolvpol	-34.8527	deltaGlgsolvpol	-35.0992	deltaGlgsolvpol	-35.11
deltaGlgsolvnonpol	6.73492	deltaGlgsolvnonpol	6.62188	deltaGlgsolvnonpol	6.59667
deltaGvdw	-107.636	deltaGvdw	-100.051	deltaGvdw	-117.937
deltaGelec	0	deltaGelec	0	deltaGelec	0
deltaG	-10.1147	deltaG	-9.88414	deltaG	-10.7462
Cluster	1	Cluster	0	Cluster	0
ClusterRank	0	ClusterRank	0	ClusterRank	0

<u>6-eNOS(L-Arginine)</u>		<u>6-nNOS(L-Arginine)</u>		<u>6-iNOS(L-Arginine)</u>	
Energy	-28.443	Energy	-23.4341	Energy	-28.027
SimpleFitness	-28.443	SimpleFitness	-23.4341	SimpleFitness	-28.027
FullFitness	-2032.56	FullFitness	-2200.21	FullFitness	-2239.06
InterFull	-99.7369	InterFull	-87.646	InterFull	-107.325
IntraFull	1.37044	IntraFull	4.22401	IntraFull	5.20546
solvFull	-2232.34	solvFull	-2431.51	solvFull	-2442.02
surfFull	298.145	surfFull	314.72	surfFull	305.081
extraFull	0	extraFull	0	extraFull	0
deltaGcompsolvpol	-2232.34	deltaGcompsolvpol	-2431.51	deltaGcompsolvpol	-2442.02
deltaGcompsolvnonpol	298.145	deltaGcompsolvnonpol	314.72	deltaGcompsolvnonpol	305.081
deltaGprotolvpol	-2268.57	deltaGprotolvpol	-2460.55	deltaGprotolvpol	-2486.33
deltaGprotolvnonpol	299.405	deltaGprotolvnonpol	315.706	deltaGprotolvnonpol	306.538
deltaGlignolvpol	-33.2622	deltaGlignolvpol	-35.9563	deltaGlignolvpol	-36.7225
deltaGlignolvnonpol	4.62845	deltaGlignolvnonpol	4.58891	deltaGlignolvnonpol	4.60597
deltaGvdw	-99.7369	deltaGvdw	-87.646	deltaGvdw	-107.325
deltaGelec	0	deltaGelec	0	deltaGelec	0
deltaG	-9.72924	deltaG	-8.83092	deltaG	-9.75731
Cluster	0	Cluster	0	Cluster	0
ClusterRank	0	ClusterRank	0	ClusterRank	0
<u>6-eNOS(1400W)</u>		<u>6-nNOS(1400W)</u>		<u>6-iNOS(1400W)</u>	
Energy	-29.8121	Energy	-26.2413	Energy	-27.8916
SimpleFitness	-29.8121	SimpleFitness	-26.2413	SimpleFitness	-27.8916
FullFitness	-4001.77	FullFitness	-2517.46	FullFitness	-4311.71
InterFull	-99.7834	InterFull	-99.2474	InterFull	-106.04
IntraFull	0.384028	IntraFull	4.97197	IntraFull	4.32722
solvFull	-4417.99	solvFull	-2736.95	solvFull	-4813.67
surfFull	515.623	surfFull	313.767	surfFull	603.676
extraFull	0	extraFull	0	extraFull	0
deltaGcompsolvpol	-4417.99	deltaGcompsolvpol	-2736.95	deltaGcompsolvpol	-4813.67
deltaGcompsolvnonpol	515.623	deltaGcompsolvnonpol	313.767	deltaGcompsolvnonpol	603.676
deltaGprotolvpol	-4451.84	deltaGprotolvpol	-2768.03	deltaGprotolvpol	-4850.39
deltaGprotolvnonpol	516.682	deltaGprotolvnonpol	314.623	deltaGprotolvnonpol	604.61
deltaGlignolvpol	-32.9622	deltaGlignolvpol	-36.2637	deltaGlignolvpol	-36.1445
deltaGlignolvnonpol	4.64535	deltaGlignolvnonpol	4.61525	deltaGlignolvnonpol	4.60484
deltaGvdw	-99.7834	deltaGvdw	-99.2474	deltaGvdw	-106.04
deltaGelec	0	deltaGelec	0	deltaGelec	0
deltaG	-9.88026	deltaG	-9.77494	deltaG	-10.086
Cluster	0	Cluster	0	Cluster	0
ClusterRank	0	ClusterRank	0	ClusterRank	0

<u>LArg-eNOS(L-Arginine)</u>		<u>LArg-nNOS(L-Arginine)</u>		<u>LArg-iNOS(L-Arginine)</u>	
Energy	-64.2624	Energy	-53.87	Energy	-73.5701
SimpleFitness	-64.2624	SimpleFitness	-53.87	SimpleFitness	-73.5701
FullFitness	-2152.13	FullFitness	-2309.79	FullFitness	-2364.76
InterFull	-110.135	InterFull	-75.9569	InterFull	-130.404
IntraFull	-110.414	IntraFull	-114.188	IntraFull	-109.599
solvFull	-2230.04	solvFull	-2434.64	solvFull	-2429.92
surfFull	298.455	surfFull	314.997	surfFull	305.162
extraFull	0	extraFull	0	extraFull	0
deltaGcompsolvpol	-2230.04	deltaGcompsolvpol	-2434.64	deltaGcompsolvpol	-2429.92
deltaGcompsolvnonpol	298.455	deltaGcompsolvnonpol	314.997	deltaGcompsolvnonpol	305.162
deltaGprotsolvpol	-2268.57	deltaGprotsolvpol	-2460.55	deltaGprotsolvpol	-2486.33
deltaGprotsolvnonpol	299.405	deltaGprotsolvnonpol	315.706	deltaGprotsolvnonpol	306.538
deltaGlgsolvpol	-43.3759	deltaGlgsolvpol	-39.4593	deltaGlgsolvpol	-47.216
deltaGlgsolvnonpol	5.64788	deltaGlgsolvnonpol	5.78154	deltaGlgsolvnonpol	5.58371
deltaGvdw	-110.135	deltaGvdw	-75.9569	deltaGvdw	-130.404
deltaGelec	0	deltaGelec	0	deltaGelec	0
deltaG	-10.0203	deltaG	-7.78892	deltaG	-10.6427
Cluster	0	Cluster	11	Cluster	1
ClusterRank	0	ClusterRank	3	ClusterRank	0
<u>LArg-eNOS(1400W)</u>		<u>LArg-nNOS(1400W)</u>		<u>LArg-iNOS(1400W)</u>	
Energy	-65.217	Energy	-59.2048	Energy	-69.241
SimpleFitness	-65.217	SimpleFitness	-59.2048	SimpleFitness	-69.241
FullFitness	-4119.37	FullFitness	-2627.31	FullFitness	-4434.49
InterFull	-92.7332	InterFull	-84.3217	InterFull	-113.143
IntraFull	-119.275	IntraFull	-120.056	IntraFull	-116.567
solvFull	-4423.24	solvFull	-2736.48	solvFull	-4808.41
surfFull	515.88	surfFull	313.547	surfFull	603.634
extraFull	0	extraFull	0	extraFull	0
deltaGcompsolvpol	-4423.24	deltaGcompsolvpol	-2736.48	deltaGcompsolvpol	-4808.41
deltaGcompsolvnonpol	515.88	deltaGcompsolvnonpol	313.547	deltaGcompsolvnonpol	603.634
deltaGprotsolvpol	-4451.84	deltaGprotsolvpol	-2768.03	deltaGprotsolvpol	-4850.39
deltaGprotsolvnonpol	516.682	deltaGprotsolvnonpol	314.623	deltaGprotsolvnonpol	604.61
deltaGlgsolvpol	-37.7024	deltaGlgsolvpol	-36.4367	deltaGlgsolvpol	-41.3568
deltaGlgsolvnonpol	5.56338	deltaGlgsolvnonpol	5.55297	deltaGlgsolvnonpol	5.51108
deltaGvdw	-92.7332	deltaGvdw	-84.3217	deltaGvdw	-113.143
deltaGelec	0	deltaGelec	0	deltaGelec	0
deltaG	-9.30704	deltaG	-8.43314	deltaG	-10.2068
Cluster	0	Cluster	3	Cluster	0
ClusterRank	0	ClusterRank	0	ClusterRank	0

<u>1400W-eNOS(L-Arginine)</u>		<u>1400W-nNOS(L-Arginine)</u>		<u>1400W-iNOS(L-Arginine)</u>	
Energy	-35.436	Energy	-34.8197	Energy	-69.0512
SimpleFitness	-35.436	SimpleFitness	-34.8197	SimpleFitness	-69.0512
FullFitness	-2066.4	FullFitness	-2248.99	FullFitness	-2407.18
InterFull	-132.891	InterFull	-140.361	InterFull	-128.601
IntraFull	-0.95525	IntraFull	-0.96852	IntraFull	-148.415
solvFull	-2230.84	solvFull	-2422.19	solvFull	-2435.34
surfFull	298.287	surfFull	314.533	surfFull	305.174
extraFull	0	extraFull	0	extraFull	0
deltaGcompsolvpol	-2230.84	deltaGcompsolvpol	-2422.19	deltaGcompsolvpol	-2435.34
deltaGcompsolvnonpol	298.287	deltaGcompsolvnonpol	314.533	deltaGcompsolvnonpol	305.174
deltaGprotolvpol	-2259.47	deltaGprotolvpol	-2460.55	deltaGprotolvpol	-2486.33
deltaGprotolvnonpol	299.42	deltaGprotolvnonpol	315.706	deltaGprotolvnonpol	306.538
deltaGlignolvpol	-74.4494	deltaGlignolvpol	-74.8673	deltaGlignolvpol	-50.3104
deltaGlignolvnonpol	4.94903	deltaGlignolvnonpol	4.94715	deltaGlignolvnonpol	6.23611
deltaGvdw	-132.891	deltaGvdw	-140.361	deltaGvdw	-128.601
deltaGelec	0	deltaGelec	0	deltaGelec	0
deltaG	-10.3684	deltaG	-8.6376	deltaG	-10.675
Cluster	0	Cluster	0	Cluster	0
ClusterRank	0	ClusterRank	0	ClusterRank	0
<u>1400W-eNOS(1400W)</u>		<u>1400W-nNOS(1400W)</u>		<u>1400W-iNOS(1400W)</u>	
Energy	-34.872	Energy	-21.4019	Energy	-38.1473
SimpleFitness	-34.872	SimpleFitness	-21.4019	SimpleFitness	-38.1473
FullFitness	-4044.91	FullFitness	-2548.67	FullFitness	-4356.38
InterFull	-129.634	InterFull	-135.898	InterFull	-149.225
IntraFull	-3.77579	IntraFull	1.55357	IntraFull	-0.85389
solvFull	-4427.14	solvFull	-2727.58	solvFull	-4809.8
surfFull	515.644	surfFull	313.254	surfFull	603.499
extraFull	0	extraFull	0	extraFull	0
deltaGcompsolvpol	-4427.14	deltaGcompsolvpol	-2727.58	deltaGcompsolvpol	-4809.8
deltaGcompsolvnonpol	515.644	deltaGcompsolvnonpol	313.254	deltaGcompsolvnonpol	603.499
deltaGprotolvpol	-4451.84	deltaGprotolvpol	-2768.03	deltaGprotolvpol	-4850.39
deltaGprotolvnonpol	516.682	deltaGprotolvnonpol	314.623	deltaGprotolvnonpol	604.61
deltaGlignolvpol	-74.7406	deltaGlignolvpol	-76.9882	deltaGlignolvpol	-75.6977
deltaGlignolvnonpol	4.99946	deltaGlignolvnonpol	5.02161	deltaGlignolvnonpol	4.95016
deltaGvdw	-129.634	deltaGvdw	-135.898	deltaGvdw	-149.225
deltaGelec	0	deltaGelec	0	deltaGelec	0
deltaG	-10.3788	deltaG	-10.2633	deltaG	-11.5632
Cluster	0	Cluster	1	Cluster	0
ClusterRank	0	ClusterRank	1	ClusterRank	2

Figure A-6. Docking parameters of **5**, **6**, L-arginine and 1400W with NOS isozymes (crystal structure in parenthesis) using Swissdock.ch and UCSF Chimera.