Hypothiocyanous Acid Oxidation of Tubulin Cysteines Inhibits Microtubule Polymerization

Hillary Meghan Clark
College of William and Mary

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Hypothiocyanous acid oxidation of tubulin cysteines inhibits microtubule polymerization

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Chemistry from The College of William and Mary

by

Hillary Meghan Clark

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(Honors)

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Williamsburg, VA
April 27, 2011
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<tr>
<td>BME</td>
<td>$\beta$-mercaptoethanol</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EPO</td>
<td>Eosinophil peroxidase</td>
</tr>
<tr>
<td>GR</td>
<td>Glutathione reductase</td>
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<td>GRS</td>
<td>Glutathione/glutaredoxin reductase system</td>
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<tr>
<td>Grx</td>
<td>glutaredoxin</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione (reduced)</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione (oxidized)</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>IAF</td>
<td>5-idoacetamidofluorescein</td>
</tr>
<tr>
<td>LPO</td>
<td>Lactoperoxidase</td>
</tr>
<tr>
<td>MAP</td>
<td>Microtubule associated protein</td>
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<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>Msr</td>
<td>Methionine sulfoxide reductase</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<td>TRS</td>
<td>Thioredoxin reductase system</td>
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Chemical Index

- Hypothiocyanous acid
- Glycine-chloramine
- Hydrogen peroxide
- Peroxynitrite
- Sodium hypochlorite
- Cyanogen bromide
- Formic acid
- Ammonium hydroxide
- Dithiothreitol (DTT)
- Luminol
5-thio-2-nitrobenzoic acid (TNB)  5-5’-disthiobis(2-nitrobenzoic acid) (DTNB)

Glutathione

5-idoacetaminofluorescein (IAF)

2-mercaptoethanol  guanidine hydrochloride
Introduction

Oxidative stress occurs in cells when there is an imbalance between the production of reactive oxygen species (ROS) and the ability of antioxidants to remove them.\(^1\) Under normal conditions, ROS are necessary and an integral part of a cell’s redox signaling pathways. However, at high concentrations, ROS have the potential to cause oxidative damage. Typically, cells are able to combat the deleterious effects of oxidants by employing antioxidant defenses, which include enzymes and small molecules. When antioxidants are not present in high enough concentrations, oxidative damage to biological molecules such as proteins, DNA, RNA, lipids, and carbohydrates can occur. Antioxidants become overwhelmed by ROS, and the system is said to be under oxidative stress.\(^1,2\) Accumulation of oxidative damage plays a role in aging and is the underlying cause of many diseases, including heart disease, many neurodegenerative diseases, diabetes, cancer, and inflammatory diseases.\(^2\) Understanding the causes and effects of oxidative modifications to biomolecules is essential to understanding many diseases and the aging process.

Reactive Oxygen Species

The formation of ROS in cells occurs during the reduction of molecular oxygen to water.\(^2\) A radical is any atom or molecule with an unpaired electron, which makes it extremely reactive. Molecular oxygen has two electrons that are not spin paired, and reside in their own discrete orbitals, making it a diradical. The distribution of electrons in oxygen makes it impossible for the molecule to accept a spin-matched pair of electrons unless one of the electrons undergoes a spin reversal, making pairing possible.\(^3\)
Therefore, molecular oxygen can only accept one electron at a time, producing a myriad of reactive oxygen species during the reduction process (Figure 1).²,⁴

Upon the addition of one electron to oxygen, the superoxide anion (O₂⁻) is formed. It is a weak oxidizing agent, but serves as a source for the production of strong oxidizing agents. The superoxide radical causes considerable damage to the phospholipid component of the cell membrane, and when two O₂⁻ are reacted, hydrogen peroxide (H₂O₂) is produced.⁴

Hydrogen peroxide is not a radical, and therefore has a more limited reactivity than many radical species. Hydrogen peroxide is able to diffuse across membranes and throughout cells fairly easily. Hydrogen peroxide is an oxidizing agent itself, and it can be especially dangerous when it reacts with transition metal ions to carry out Fenton chemistry, which produces radicals. The reaction of H₂O₂ with transition metals produces the highly reactive hydroxyl radical (·OH) and the hydroxide ion.

The hydroxyl radical is perhaps the most dangerous ROS since it can initiate radical chain reactions, which cause considerable damage. Once a hydroxyl radical is formed, it only travels a short distance before reacting with nearly any biomolecule with which it comes in contact. Lipids, found in cell membranes, are especially prone to damage by radical chain reactions, which do not stop until a radical quenching antioxidant is present.⁴ Finally, the last step of the reduction of oxygen occurs as an electron is added to ·OH to give water.²,⁴
Figure 1: Formation of reactive oxygen species. The four electron reduction of molecular oxygen to form two molecules of water.  

Reactive oxygen species can originate from cellular sources other than the reduction of molecular oxygen. ROS can also be generated when electrons are leaked from the electron transport chain and form superoxide. Another source of ROS in cells is the biotransformation of xenobiotics and the respiratory burst in white blood cells. In this case, ROS are purposefully generated by the cell to kill a pathogen, and ROS act a major part of cellular defense.

Neutrophils, which are immune cells found in blood, produce oxidants to protect organisms from microbial pathogens. Neutrophils are phagocytes, meaning they are capable of engulfing harmful prey such as bacteria. Upon endocytosis, an engulfed bacterium fuses with lysosomes and the cell’s oxygen consumption increases nearly 100-fold. This respiratory burst occurs when the enzyme NADPH oxidase converts molecular oxygen to superoxide. Next, superoxide dismutase combines two superoxides
and to form hydrogen peroxide, which can react to form other oxidative species and kill bacteria.\textsuperscript{4}

Neutrophils also contain the enzyme myeloperoxidase (MPO) which catalyzes the reaction of H\textsubscript{2}O\textsubscript{2} with halide ions to form hypohalous acids, which are oxidizing agents.\textsuperscript{5} One common example of a hypohalous acid is HOCl, or bleach, a strong bactericidal agent. Superoxide and hydrogen peroxide can also react to form the hydroxyl radical. Nitric oxide can react with superoxide to form peroxynitrite (ONOO\textsuperscript{-}), a strong oxidizing agent. The various reactive species directly damage bacterial contents and also activate cellular proteases, which destroy bacterial proteins. Lysosomes then recycle and digest the remaining materials.\textsuperscript{4} In the case of respiratory burst, the body is deliberately producing reactive oxygen species to overcome the invasion of pathogens, but the oxidants can also become harmful by reacting with and damaging non-bacterial proteins, nucleotides, lipids, and carbohydrates.

High exposure to oxidants has deleterious effects, but moderate exposure to oxidants is normal and necessary. Often, cellular signaling responses rely on moderate oxidative modifications. When the redox homeostasis of a cell is altered, it leads to downstream effects which can include the increased expression of repair enzymes.\textsuperscript{6} Cells depend on moderate amounts of oxidants as a way to monitor the activity of transcription factors, membrane channels, metabolic enzymes, as well as alter calcium-dependent and phosphorylation signaling pathways.\textsuperscript{6} By slightly changing the redox status of certain proteins, ROS are an important part of cell signaling, metabolic pathways, and transcription factors.\textsuperscript{4,6} It is important to recognize the necessity of oxidants for normal
cellular signaling and function, while remembering that ROS also have damaging biological effects when found in high concentrations.

**Antioxidants**

Living organisms have evolved numerous ways to protect themselves from the dangers of oxidative stress, including antioxidant enzymes and molecules. Antioxidants are defined by Gutteridge\(^7\) as any substance that, when present at low concentrations, compared to those of the oxidizable substrate, considerably delays or inhibits oxidation of the substrate. Common cellular antioxidants include the enzymes superoxide dismutase, glutathione peroxidase, catalase, the small tripeptide glutathione (GSH), as well as vitamin C and vitamin E.\(^1,^4\) Superoxide dismutase (SOD) catalyzes the removal of two \(\text{O}_2^\cdot\) to form \(\text{H}_2\text{O}_2\) and molecular oxygen. A dismutation reaction is a reaction in which the same element is both oxidized and reduced. In the reaction catalyzed by superoxide dismutase, one \(\text{O}_2^\cdot\) is oxidized to form molecular oxygen, while the other is reduced to form hydrogen peroxide.

\[
2\text{O}_2^\cdot + 2\text{H}^+ \overset{\text{SOD}}{\longrightarrow} \text{H}_2\text{O}_2 + \text{O}_2
\]

There are multiple pathways for cellular hydrogen peroxide, a molecule which is free to diffuse throughout the cell. It can react to form more reactive oxygen species, as explained above, or be degraded enzymatically. One way hydrogen peroxide can react is to form the hydroxyl radical. It reacts with transition metal ions to form hydroxyl radicals, in a reaction known as Fenton chemistry.\(^1,^4\) While this reaction removes hydrogen peroxide, it creates the reactive hydroxyl radical, so it is not considered an antioxidant reaction.
Hydrogen peroxide can also be degraded enzymatically, which is an antioxidant defense. Glutathione peroxidase catalyzes the reduction of $\text{H}_2\text{O}_2$ to water which requires two reduced glutathione molecules (GSH). GSH is a tripeptide containing the amino acids cysteine, glutamic acid, and glycine, and is a major participant in antioxidant defenses. As hydrogen peroxide is reduced to water, two molecules of GSH are oxidized, forming a disulfide $^{1,6}$:

$$\text{GSH peroxidase} \quad 2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O}$$

Catalase is an antioxidant enzyme present in peroxisomes, and its primary role is to catalyze the reduction of two $\text{H}_2\text{O}_2$ to form water and dioxygen.$^{1,4}$ Catalase contains a heme group which participates in the redox reaction necessary to remove $\text{H}_2\text{O}_2$:

$$\text{Catalase} \quad 2\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O}$$

Vitamin E ($\alpha$-Tocopherol) and Vitamin C (ascorbate) act as antioxidants in cells. Vitamin E is a radical scavenger and is known as a phenolic antioxidant. Phenolic antioxidants are useful because their radical products are resonance stabilized by the phenol ring.$^4$ Vitamin E is lipophilic, and therefore it is found in cell membranes where it helps prevent oxidative damage to lipids.$^1$ Vitamin E acts as a radical scavenger, and is capable of disrupting the radical chain reaction of lipid peroxidation.$^1$ Figure 2 shows the lipophilic structure of vitamin E and how radicals are resonance stabilized by a phenol ring. The resonance stabilization helps to quench radicals, and terminate radical chain reactions.
Figure 2: Vitamin E and phenolic stabilization of radicals. a) Structure of α-tocopherol, or vitamin E. b) resonance stabilization of a radical by a phenol ring.

Vitamin C, or ascorbic acid, is a water soluble antioxidant and is responsible for quenching free radicals as well as regenerating the reduced form of vitamin E. Vitamin C undergoes two 1 electron oxidation steps. Ascorbate scavenges a variety of radical species in the cytoplasm, preventing damage to the membrane. After regenerating the reduced form of vitamin E, ascorbate is regenerated by reacting with GSH. Vitamin C is reversibly oxidized and reduced as seen in Figure 3.

Cysteine Oxidation and Repair

All amino acids can be oxidatively modified, but the sulfur containing amino acids, cysteine and methionine, are especially susceptible to oxidation. Cysteine and
methionine are also the only amino acids that can be enzymatically repaired back to the reduced forms. The oxidation of cysteine leads to a variety of oxidized products, including sulfenic acid, sulfonic acid, inter- and intra-molecular disulfides, mixed disulfides, and thiyl radicals. The major way for reactive oxygen species to integrate themselves into cellular signal transduction pathways is through redox reactions with the thiol (RSH) group of cysteine.

Under normal conditions, the redox state of critical thiols is believed to play a role in normal metabolism, with the oxidized or reduced forms corresponding to an active or inactive protein. When cells are under oxidative stress, redox signaling is initiated and leads to a number of downstream effects, such as increased expression of repair enzymes. However, oxidizing protein thiols can also result in damage to proteins, with severe functional consequences.

There are two major proposed mechanisms for redox signaling. One is thermodynamic in nature and is based on the idea that all thiols and disulfides exist in equilibrium with each other. In this model, glutathione (GSH) is the redox buffer, and exists in its oxidized or reduced form based on the redox potential of the cell. The other proposed mechanism is based on the kinetic properties of specific, sensitive targets. It is well established that cells respond to oxidative stress by altering the redox state of critical thiols.

Nearly all physiological oxidants react with thiols, although most show selectivity for the thiolate anion. The free amino acid cysteine has a pKₐ of 8.3, the thiol on GSH has a pKₐ of 8.8, and the pKₐ values for protein thiols depend upon the molecular environment of the protein. Oxidants can react with thiols by causing one electron or
two electron oxidations. The one electron oxidation forms a thyl radical, which undergoes further reactions including transferring the radical to the antioxidant ascorbate. The thyl radical preferentially reacts with a thiolate anion from a protein or glutathione to form the disulfide anion radical. The two electron oxidation of thiols initially produces sulfenic acid (RSOH) which also participates in a variety of further reactions, producing sulfinic acid, sulfonic acid, sulfanamide, sulfanamide, sulfonylamide, mixed disulfides, and inter- and intra-molecular disulfides. The one and two electron oxidations of protein thiols are summarized in Figure 4.

Figure 4: Oxidation products of protein thiols. Both the 1 and 2 electron oxidations can result in glutathionylation and protein disulfides. (Pr = protein)

Almost all physiologically relevant oxidants are capable of reacting with thiols; however, they react in different ways. Some oxidants are weak oxidants, while others have a high specificity for thiols. H₂O₂ is an oxidizing agent that reacts directly with thiolate anions, following the two electron oxidation path illustrated in Figure 4. It is produced in cells as a secondary product of the superoxide produced by NADPH oxidases, and non-intentionally as a by-product of regular metabolic processes.
Oxyradicals cover a wide range of radical oxygen species, most of which react well with thiols. Highly reactive radical species, such as the hydroxyl radical, are likely not involved in signal transmission since they react indiscriminately with all biomolecules. Other oxyradicals are less reactive than the hydroxyl radical and more specific, so they could be involved with redox signaling and not just oxidative damage. Radical oxidation reactions can produce thyl and sulfinyl radicals during the process of a radical chain reaction. Other common thiol oxidants include peroxynitrite, which can react directly with thiols or break down into the reactive hydroxyl radical and nitrogen dioxide, and hypohalous acids such as HOCl and HOBr. All of these species react with cysteines as well as other amino acids.

Since cysteine oxidation is reversible, it is suggested that the oxidation state of cysteine can play a regulatory role, analogous to the phosphorylation and dephosphorylation regulation of protein activity. The most straightforward redox signaling mechanism is the inactivation of an enzyme upon the oxidation of cysteine residues in the active site. Many enzymes require reduced cysteine residues in the active site to help orient the substrates and catalyze the reaction, so if a cysteine in an active site is oxidized, it would likely render the enzyme inactive. When intra-molecular disulfide bonds form, they can cause significant changes in a protein’s tertiary structure, and the shape changes can affect the functional properties of the protein.

There are two known repair mechanisms for cysteine, the thioredoxin reductase system and the glutathione/glutaredoxin reductase system. Thioredoxin and glutaredoxin are both small proteins containing a redox-active disulfide in the active site. The goal of these systems is to reduce critical cysteine residues in damaged proteins.
The thioredoxin reductase system (TRS) reverses small molecule and protein disulfides, and it is composed of thioredoxin reductase (TrxR), thioredoxin (Trx), and NADPH.\textsuperscript{11,12} TrxR functions by catalyzing the transfer of electrons from NADPH to reduce the active site disulfide of oxidized Trx, Trx-\(S_2\), resulting in the dithiol, Trx-(SH)\(_2\). Next, the reduced form of Trx is oxidized as it reduces the disulfide of the protein substrate.\textsuperscript{12,13}

\[
\text{NADPH} + \text{H}^+ + \text{Trx-S}_2 \rightarrow \text{NADP}^+ + \text{Trx-(SH)}_2 \\
\text{Trx-(SH)}_2 + \text{protein-S}_2 \rightarrow \text{Trx-S}_2 + \text{protein-(SH)}_2
\]

Figure 5: Thioredoxin reductase system. The TrxR/Trx system showing substrates for Trx, including oxidized proteins and some transcription factors. Abbreviations: Trx-(SH)\(_2\) and Trx-S\(_2\), reduced and oxidized thioredoxin respectively.

The glutathione/glutaredoxin reductase system (GRS) is also capable of reducing protein disulfides and is composed of glutathione reductase (GR), glutaredoxin (Grx), glutathione (GSH), and NADPH.\textsuperscript{11} It behaves similarly to the thioredoxin reductase system and undergoes thiol/disulfide exchange. First a protein disulfide is reduced to a dithiol by Grx. The oxidized form of Grx undergoes thiol/disulfide exchange with GSH to yield the dithiol form of Grx and oxidized GSSG. In the third step, GSSG is reduced back to GSH by GR, using NADPH for electron transfer.\textsuperscript{14}
Methionine Oxidation and Repair

Other than cysteine, methionine is the only amino acid with an enzymatic repair mechanism. Methionine is a thioether, and is readily oxidized by a variety of oxidizing agents. The side chain of methionine is long, flexible, and non-polar, and therefore methionine residues are typically found in the hydrophobic interiors of folded proteins, although some proteins have exposed methionine residues.

When methionine is oxidized, the protein undergoes conformational changes which, along with the reversibility of methionine sulfoxide, suggests a role for methionine in reversible signaling pathways. It is well established that cysteine residues participate in redox signaling pathways, but the oxidation and reduction of methionine is a more recently discovered molecular mechanism for cellular signaling and regulation.

The oxidation of methionine in many proteins inactivates the protein, but there is evidence that the oxidation of exposed, surface methionine residues does not inhibit protein function. This suggests, under some circumstances, that methionine has local...
antioxidant capacity. However, the oxidation of methionine residues can also damage proteins in such a way that enzymatic repair is not possible.

When methionine is oxidized by two electrons, methionine sulfoxide is formed. This first product of methionine oxidation can be repaired by the methionine sulfoxide reductase system. When methionine is further oxidized, it forms methionine sulfone, which cannot be enzymatically repaired.

![Figure 7: Oxidation of methionine to methionine sulfoxide and methionine sulfone.](image)

Methionine is oxidized by many of the same physiologically relevant ROS as cysteine, such as hydrogen peroxide, peroxynitrite, hypohalous acids, the hydroxyl radical, and haloamines. All of the oxidants mentioned oxidize methionine to methionine sulfoxide, and if the oxidant is present in high enough concentrations, methionine sulfoxide is irreversibly oxidized to methionine sulfone.

Just as oxidized cysteines are reduced by the thioredoxin reductase system and the glutathione/glutaredoxin system, methionine sulfoxide can be reduced to methionine by methionine sulfoxide reductase A (MsrA) or methionine sulfoxide reductase B (MsrB), which reduce the S-epimer or the R-epimer, respectively. The Msr system is composed of methionine sulfoxide reductase, thioredoxin, thioredoxin reductase, and NADPH.
is only capable of reducing methionine sulfoxide to methionine, it is not able to reduce methionine sulfone. Figure 8 summarizes the necessary components of the Msr system and how the components interact.

Figure 8: Methionine sulfoxide reductase. Oxidation and reduction of methionine residues, showing the methionine sulfoxide reductase system (Msr). Methionine is easily oxidized to methionine sulfoxide, and can be reduced back to methionine by the enzyme MsrA. Under physiological conditions, MsrA is coupled to the cellular redox system through thioredoxin, thioredoxin reductase, and NADPH.

Tubulin, Microtubules, and Alzheimer’s Disease

Tubulin is a protein that polymerizes to form the ubiquitous, vital cell components known as microtubules. Microtubules compose part of the eukaryotic cytoskeleton, and have a variety of cellular functions including separation of chromosomes during mitosis and the transport of vesicles. Microtubules create a cellular scaffolding system to help
maintain cell shape, and they are especially important in neurons because of the need for extensive intracellular trafficking over long distances. Microtubules are found in higher concentrations in the brain than in any other tissue.\textsuperscript{18}

Tubulin is a heterodimer, composed of two similar 50,000 kDa subunits $\alpha$-tubulin and $\beta$-tubulin. Both $\alpha$- and $\beta$-tubulin are highly conserved proteins.\textsuperscript{19,20} The microtubule core is hollow and cylindrical in shape with a diameter of about 25 nm, and it is formed by 13 tubulin protofilaments (Figure 9). Microtubule protofilaments are formed by the head-to-tail polymerization of $\alpha$- and $\beta$-tubulin heterodimers. Microtubules are polar structures, consisting of a fast-growing plus end and a slow-growing minus end.\textsuperscript{19}

![Figure 9: Microtubule structure. Tubulin heterodimers forming microtubules.](image)

Microtubule assembly follows the classical view of biopolymer assembly, meaning that it consists of two main steps, nucleation and elongation.\textsuperscript{19} Microtubule nucleation and elongation depend on external energy in the form of guanosine
triphosphate (GTP), which is bound to tubulin and hydrolyzed to GDP upon microtubule assembly.\textsuperscript{19} Disassembly of microtubules is independent of external energy sources. Eukaryotic microtubules are labile polymers, meaning they quickly exchange subunits with the pool of soluble tubulin heterodimers.\textsuperscript{18} Tubulin subunits and microtubules seem to exist in a state of equilibrium, assembling and disassembling in a treadmill-like fashion.\textsuperscript{20} The steady-state microtubule preferentially adds subunits at the plus end and loses them at the minus end, maintaining the length of the microtubule. It is also possible that microtubules go through periods of growth and shrinkage, and do not maintain a constant length.\textsuperscript{19}

Microtubules do not exist as independent structural components in cells. Instead, they interact with an assortment of other proteins known as microtubule-associated proteins (MAPs). MAPs play an important role in the assembly and stability of microtubules, as well as aid in the interaction of microtubules with organelles.\textsuperscript{18,21} The most commonly studied MAPs are MAP1, MAP2, and tau.

Higher eukaryotes contain multiple genes for $\alpha$-tubulin and $\beta$-tubulin, and the concentration of tubulin in cells depends upon tissue type.\textsuperscript{19,22} Tubulin comprises 12-14\% of the total cellular protein concentration in mammalian brain tissue and 2-4\% of total protein concentration in nonneuronal cells.\textsuperscript{22} Tubulin has 20 free cysteines (12 in $\alpha$-tubulin and 8 in $\beta$-tubulin), which are especially prone to oxidative damage. The high concentration of tubulin in neurons, coupled with the large number of reduced cysteine residues, makes tubulin a likely target for attack by ROS. If the oxidation of tubulin cysteine residues is easily repaired by either the TRS or GRS, it is possible that tubulin cysteines act as a redox buffer to protect other vulnerable proteins and amino acids from
oxidative damage. It is important to understand the effects of oxidants on tubulin, as it could lead to a better understanding of diseases caused by the accumulation of oxidative modifications.

Oxidative damage to proteins and other biomolecules has been implicated as a cause of Alzheimer’s disease and other neurodegenerative diseases.\textsuperscript{23,24} The production of oxidized proteins and formation of oxidation-mediated protein aggregation contributes to both the normal and pathological aging processes.\textsuperscript{24} Abnormalities of the cytoskeleton have been detected in Alzheimer’s brain tissue. Since tubulin and MAPs are essential parts of the neuronal cytoskeleton and required for proper neuron function, Dr. Landino’s lab is interested in determining the effects of oxidation on tubulin.

Previous work in Dr. Landino’s lab showed that when bovine brain tubulin is treated with peroxynitrite (ONOO\textsuperscript{–}), tubulin thiols are oxidized to disulfides and polymerization is inhibited in a dose-dependent fashion.\textsuperscript{12,25} When the damaged tubulin samples were treated with disulfide reducing agents, such as dithiothreitol (DTT) or β-mercaptoethanol (BME), a significant portion of polymerization activity was restored. In another study, it was determined that both the TRS and GRS repaired tubulin disulfides and restored a significant portion of polymerization activity.\textsuperscript{12} Peroxynitrite-induced oxidation of tubulin cysteines to disulfide bonds is at least partially responsible for the inhibition of microtubule assembly.\textsuperscript{12,25}

Since the initial ONOO\textsuperscript{–} work was reported in 2001, many other oxidants have been shown to modify tubulin cysteines and inhibit tubulin polymerization activity. These include HOCl, chloramines, nitric oxide donors, and nitroxyl donors.\textsuperscript{25-27} All of these oxidants modify cysteine residues, but they also show some reactivity with other
commonly oxidized amino acids, such as methionine, tyrosine, lysine, tryptophan, and histidine.\textsuperscript{10} Therefore, a thiol specific oxidant would be a useful tool to probe the effect of cysteine oxidation only on tubulin polymerization activity.

**Hypothiocyanous Acid**

As mentioned previously, part of immune cells’ natural defense system is to produce hypohalous acids by the peroxidase enzyme catalyzed reaction of H\textsubscript{2}O\textsubscript{2} with halide ions. The hypohalous acids produced by myeloperoxidase (MPO) and eosinophil peroxidase (EPO), such as HOCl and HOBr, are important bacteriocidal agents, but they can also lead to tissue inflammation and damage. Hypothiocyanous acid (HOSCN) is considered a pseudohypohalous acid, since the thiocyanate anion (SCN\textsuperscript{−}) is a pseudohalide, and is also produced by peroxidase enzymes in cells. HOSCN is a milder oxidizing agent than HOCl or HOBr, but it has an extremely high specificity for thiols. Therefore, it could be a more potent inducer of cell death than HOCl or HOBr because it targets critical cysteine residues.\textsuperscript{28}

Physiological concentrations of the different halide ions and the specificity of the peroxidase enzymes for the halide ion substrates determine the concentration of hypohalous acids produced by peroxidases in white blood cells (provided there is H\textsubscript{2}O\textsubscript{2} present). Physiological concentrations of relevant halide and pseudohalide ions are as follows: 100-140 mM chloride, 20-100 \( \mu \)M bromide, less than 1 \( \mu \)M iodide, and less than 120 \( \mu \)M thiocyanate.\textsuperscript{28} Even though there is approximately 1000 times as much Cl\textsuperscript{−} present as SCN\textsuperscript{−}, MPO has a higher specificity for SCN\textsuperscript{−} and it is suggested that MPO
produces equimolar concentrations of HOCl and HOSCN under physiological conditions.\textsuperscript{28,29}

MPO and EPO are found in activated phagocytes, while another enzyme, lactoperoxidase (LPO), is found in milk, saliva, and tears.\textsuperscript{28-30} The lactoperoxidase system is an antimicrobial system, and it is composed of LPO, H\textsubscript{2}O\textsubscript{2}, and SCN\textsuperscript{−}. The oxidation of SCN\textsuperscript{−} by peroxidases and H\textsubscript{2}O\textsubscript{2} to form HOSCN is believed to proceed by the following reaction mechanism:

\[
\begin{align*}
\text{H}_2\text{O}_2 & \quad + \quad 2\text{SCN}^{-} + 2\text{H}^{+} \quad \rightarrow \quad 2\text{H}_2\text{O} \quad + \quad (\text{SCN})_2 \\
(\text{SCN})_2 & \quad + \quad \text{H}_2\text{O} \quad \leftrightarrow \quad \text{HOSCN} \quad + \quad \text{H}^{+} \quad + \quad \text{SCN}^{-} \\
\text{HOSCN} & \quad \leftrightarrow \quad \text{OSCN}^{-} \quad + \quad \text{H}^{+}
\end{align*}
\]

The pK\textsubscript{a} of HOSCN is 5.3, therefore at physiological pH (7.4) there is a mixture of the protonated and anionic forms, this mixture will be called HOSCN hereon.

HOSCN decomposes rapidly to produce a myriad of relatively unknown decomposition products. While the nature of these decomposition products is controversial, it is speculated that they could include (SCN)\textsubscript{2}, HO\textsubscript{2}SCN, HO\textsubscript{3}SCN, (SCN)\textsubscript{3}−, CN\textsuperscript{−}, OCN\textsuperscript{−}, and various radical species.\textsuperscript{29} Since the identity of all the decomposition products of HOSCN is not known, and the products’ properties have not been extensively studied, it is possible that some of the decomposition products are also capable of reacting with biological molecules.

HOSCN is reported to react with thiol-containing proteins with significantly more selectivity than HOCl or HOBr. Additionally, a greater extent of apoptosis and necrosis in murine macrophage cells was observed upon treatment with HOSCN compared to treatment with equimolar amounts of HOCl or HOBr.\textsuperscript{28} HOSCN is capable of depleting
cellular glutathione in mammalian cells and inactivating thiol dependent proteins such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutathione transferases (GSTs), and membrane ATPases.\textsuperscript{28} The reaction of HOSCN with protein thiols to form disulfides is believed to proceed according to the following reactions:

\[ \text{R-SH} + \text{HOSCN} \rightarrow \text{R-S-SCN} + \text{H}_2\text{O} \]

\[ \text{R-S-SCN} + \text{R'}-\text{SH} \rightarrow \text{R-S-R'} + \text{SCN}^- + \text{H}^+ \]

It was determined that in the absence of protein thiols, or once all thiols are depleted, HOSCN is capable of oxidizing tryptophan residues.\textsuperscript{29} HOSCN-mediated oxidation of tryptophan results in several stable products, which in turn lead to protein unfolding.\textsuperscript{29} It should also be noted that HOSCN is specific for thiols, not thioethers, and therefore does not react with methionine. The selectivity of HOSCN for thiols and not thioethers makes it a useful tool for probing the effects of only cysteine oxidation.

**Explanation of analytical methods**

**Detection of cysteine oxidation**

When cysteine residues are oxidized, they no longer contain the free thiol group. Therefore, we can take advantage of 5-iodoacetamidofluorescein (IAF)—a thiol specific, fluorescein labeling reagent. Since IAF only labels reduced cysteines, as more cysteine residues are oxidized, the amount of fluorescent label incorporated into the protein decreases. The extent of cysteine oxidation can be observed qualitatively by conducting SDS-PAGE with labeled proteins, or quantitatively by measuring absorption at 490 nm. The absorption maximum of fluorescein is actually at 495 nm, but the plate reader in Dr.
Landino’s lab is equipped with a filter for 490 nm. Since all the experiments included standard curves for fluorescein, the absorption values at 490 nm were sufficient.

![Figure 10: IAF labeling of tubulin thiols. (Fl = IAF label only on reduced cysteines)](image)

**Protein Separation**

One method for separating proteins by size is SDS-PAGE, or sodium dodecyl sulfate-polyacrylamide gel electrophoresis. SDS-PAGE is often used during protein purification, to identify proteins based on molecular weight and to assess homogeneity. Sodium dodecyl sulfate (SDS) is an anionic detergent capable of binding to proteins. The interaction of SDS with proteins disrupts all noncovalent protein bonds, and the protein unfolds. Since SDS carries a negative charge, the resulting protein-SDS complex is negatively charged. SDS provides the charge needed for protein separation, but the proteins are separated by size because of the polyacrylamide matrix. Polyacrylamide is a polymer formed by acrylamide monomers, and it forms a gel matrix with intricate tunnels and pathways which vary in size. The polyacrylamide matrix allows for different sized proteins to move through the gel at different rates. When an electric current is applied to the gel, proteins, which carry a negative charge due to SDS, migrate towards the positive electrode. Smaller proteins move through the polyacrylamide matrix with more ease, so they travel farther on the gel. After separation, proteins can be visualized with a stain.
Also, prior to separation, proteins can be fluorescently labeled, which allows for easy visualization following separation.\textsuperscript{31}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{gel_electrophoresis.png}
\caption{Gel electrophoresis. SDS-PAGE separation of proteins based on molecular weight.}
\end{figure}

\textit{Protein Detection}

Western blotting is a commonly used method to detect the presence of and quantify a particular protein in a complex mixture.\textsuperscript{32} First, protein samples are separated using SDS-PAGE then they are electrophoretically transferred to a nitrocellulose or polyvinylidene fluoride (PVDF) membrane. Once the proteins have been transferred to the membrane, they are immobilized. The next step is to block the membrane to prevent interactions between the membrane and the antibody needed for the protein of interest.\textsuperscript{32} Blocking is typically done with non-fat, dry milk. Milk proteins bind to the membrane everywhere except for where target proteins are already bound, thus blocking any interactions between the membrane and antibody. If blocking is omitted, background noise is detected.

The next step is to treat the membrane with a primary antibody that has been raised to detect the target protein. After washing, the membrane is incubated with an enzyme-conjugated secondary antibody that is reactive towards the primary antibody.
Once the membrane is washed again, it is incubated with an appropriate substrate, which allows for imaging.\textsuperscript{32}

The most popular form of Western blotting involves luminol-based substrates which release light energy, called a chemiluminescent signal. Light is only produced from the reaction of the enzyme and the substrate, therefore once the substrate is depleted, no more light is emitted. Typically the enzyme is horseradish peroxidase (HRP), and the substrate is luminol. In the presence of hydrogen peroxide, HRP oxidizes luminol to form 3-aminophthalate in its excited state, which releases light as it relaxes to its ground state.\textsuperscript{32}
Materials

Porcine brains were obtained from Smithfield Packing Company (Smithfield, VA, USA). A mouse antibody against β-tubulin (monoclonal, clone TUB 2.1; 1:1 w/glycerol), 1:1 w/glycerol), the goat anti-mouse secondary antibody-horseradish peroxidase (HRP) conjugate, bovine lactoperoxidase, and rabbit muscle GAPDH were obtained from Sigma Chemicals (St. Louis, MO, USA). Catalase (30 mg/ml) from bovine liver was from Sigma Life Sciences. X-ray film, West Pico chemiluminescence detection system and 5-iodoacetamidofluorescein (IAF) were from Pierce (Rockford, IL, USA). Cyanogen bromide was from MP Biomedical (Solon, CA, USA). Dithiothreitol (DTT) was obtained from Fisher Scientific (Pittsburg, PA, USA).

Methods

Purification of tubulin

Tubulin from porcine brains was purified by Dr. Landino by two cycles of temperature-dependent polymerization and depolymerization and subsequent phosphocellulose chromatography. Tubulin in PME buffer (0.1M Pipes, pH 6.9, 1mM MgSO₄, 2mM EGTA) containing 0.1mM GTP was stored at -80°C. The tubulin used for
oxidation reactions was further purified by desalting. The concentration of tubulin was determined to be 2.65μg/μl by the bicinchoninic acid (BCA) protein assay (Pierce).

**Preparation of thionitrobenzoic acid**

Thionitrobenzoic acid (TNB) was prepared from 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) as described, although some modifications were made.\textsuperscript{33,34} DTNB (0.5g) was dissolved in 25ml 0.5M Tris-HCl, pH 8.8 and treated with 2.5ml β-mercaptoethanol. HCl (6M) was added to adjust the pH to 1.5, and the reaction was incubated at 4°C overnight. Orange crystals formed in the tube. The supernatant was discarded, and the crystals were filtered and washed with HCl (0.1M). TNB crystals were checked for purity by HPLC with a detection wavelength of 320nm and were stored at -20°C.

**Preparation and quantification of hypothiocyanous acid**

HOSCN was prepared enzymatically using lactoperoxidase (LPO) from bovine milk. LPO (12.5μg), KSCN (1mM), and H₂O₂ (0.6mM) were vortex mixed in 0.1M sodium phosphate buffer (PB) pH 6.4 (total volume 250μl). It was determined that H₂O₂ must be added last. The reaction was incubated for 15 minutes at room temperature. Catalase (10μl of 30 mg/ml) was added and the tube was mixed to remove excess H₂O₂. The reaction containing HOSCN was centrifuged (10,000g for 10 minutes, room temperature) in a 10,000 Da molecular weight cut-off Millipore centrifugation filter tube to remove LPO and catalase.

The concentration of HOSCN was assessed immediately by quantification of the consumption of TNB. A 96 well microtiter plate was used for quantification by plate
reader analysis at 405nm. A TNB standard curve was prepared containing TNB concentrations of 12.5μM-100μM in 0.1M sodium phosphate buffer pH 7.4 in a total volume of 200μl (Table 1). To quantify HOSCN, 100μM TNB was reacted with 10, 20, 30, 40, and 50μl of HOSCN, in a total volume of 200μl in sodium phosphate buffer pH 7.4 (Table 2). The absorbance values were measured by a plate reader at 405nm, and the concentration of HOSCN was determined through its stoichiometric relationship to TNB (seen in the reaction below). The average concentration of HOSCN produced from ten independent reactions was 0.215mM. The lowest concentration obtained was 0.187mM and the highest was 0.238mM.

\[
2\text{TNB} + \text{HOSCN} \rightarrow \text{DTNB} + \text{SCN}^- + \text{H}_2\text{O}
\]
(yellow) (colorless)

<table>
<thead>
<tr>
<th>V. 1mM TNB</th>
<th>V. 0.1M PB pH 7.4</th>
<th>[TNB]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0μl</td>
<td>200μl</td>
<td>Blank</td>
</tr>
<tr>
<td>2.5μl</td>
<td>197.5μl</td>
<td>12.5μM</td>
</tr>
<tr>
<td>5μl</td>
<td>195μl</td>
<td>25μM</td>
</tr>
<tr>
<td>10μl</td>
<td>190μl</td>
<td>50μM</td>
</tr>
<tr>
<td>15μl</td>
<td>185μl</td>
<td>75μM</td>
</tr>
<tr>
<td>20μl</td>
<td>180μl</td>
<td>100μM</td>
</tr>
</tbody>
</table>

Table 1: Volumes of 1mM TNB and 0.1M PB pH 7.4 added to the wells of a 96 well plate to produce a TNB standard curve with [TNB] = 12.5μM - 100μM

<table>
<thead>
<tr>
<th>V. 1mM TNB</th>
<th>V. 0.1M PB pH 7.4</th>
<th>V. HOSCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>20μl</td>
<td>170μl</td>
<td>10μl</td>
</tr>
<tr>
<td>20μl</td>
<td>160μl</td>
<td>20μl</td>
</tr>
<tr>
<td>20μl</td>
<td>150μl</td>
<td>30μl</td>
</tr>
<tr>
<td>20μl</td>
<td>140μl</td>
<td>40μl</td>
</tr>
<tr>
<td>20μl</td>
<td>130μl</td>
<td>50μl</td>
</tr>
</tbody>
</table>

Table 2: Volumes of 1mM TNB, 0.1M PB pH 7.4, and HOSCN added to the wells of a 96 well plate to determine the concentration of HOSCN.
Preparation of peroxynitrite

ONOO\(^{-}\) was prepared by Dr. Landino from acidified H\(_2\)O\(_2\) and sodium nitrite as described.\(^{34}\) The concentration of ONOO\(^{-}\) was determined by measuring the absorbance at 302 nm (\(\varepsilon_{302} = 1670\text{M}^{-1}\text{cm}^{-1}\)) in 0.1M NaOH, and it was stored at -80°C. ONOO\(^{-}\) was diluted in 0.1M NaOH immediately before it was used. To normalize the pH in oxidation reactions, NaOH was diluted to appropriate concentrations so that the same volume of ONOO\(^{-}\) was added to each oxidation reaction (ONOO\(^{-}\) solution contributed 10\% of total reaction volume).

Preparation of glycine chloramine

Glycine chloramine (5mM) was prepared immediately before use. HOCl was added to glycine in 0.1M sodium phosphate buffer pH 7.4, with a final molar ratio of HOCl to glycine 1:5.

Gel electrophoresis

Gel electrophoresis was carried out using the Mini-PROTEAN 3 cell (Bio-Rad). Gel cassette sandwiches were removed from the casting frame and placed into the electrode assembly. The electrode assembly was secured and placed in the electrophoresis mini tank. The inner chamber was filled with 300mL of 1X running buffer. Next, protein samples were loaded into the wells of the gel, and the top was placed on the tank. Gels were run at 90V for approximately 90 minutes.
Labeling of protein cysteines with IAF

Tubulin and model proteins were diluted to 100μM cysteines in 0.1M sodium phosphate buffer pH 7.4, and then treated with oxidants (25-100μM) for 20 minutes at room temperature, in a total reaction volume of 30μl. IAF in DMF (dimethylformamide) was added to the reaction to a final concentration of 1.25mM. The protein reaction was incubated with IAF for 30 minutes at 37°C. Proteins were resolved under reducing conditions by SDS-PAGE on 7.5% acrylamide gels and images were captured by camera using a UV transilluminator.

Additionally, IAF-labeled proteins were precipitated in 80% ethanol at 4°C for 30 minutes. Protein pellets were obtained by centrifugation at 12,000g for 15 minutes. Supernatant was discarded, and the protein pellet was washed twice with 80% ethanol. Protein pellets were resuspended in 200μl of 6M guanidine-HCl in PB pH 7.4, and transferred to a 96 well plate. Fluorescein standards were prepared in 6M guanidine-HCl. Absorbance values of the standards and the samples were measured at 490nm.

<table>
<thead>
<tr>
<th>Tubulin Oxidation Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tubulin (2.65μg/μl)</strong></td>
</tr>
<tr>
<td>5.7μl</td>
</tr>
<tr>
<td>5.7μl</td>
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<tr>
<td>5.7μl</td>
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<tr>
<td>5.7μl</td>
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<tr>
<td>5.7μl</td>
</tr>
</tbody>
</table>

Table 3: Volumes of tubulin, PB pH 7.4, and oxidant used in reactions that were subsequently labeled with IAF. The concentration of tubulin cysteines in each sample was 100μM, and the concentration of oxidant varied from 25-100μM. The control contained tubulin only.

Detection of higher molecular weight disulfides by Western blot

IAF labeling is useful for detecting cysteine oxidation, but it does not provide information on whether the disulfides forming are intramolecular or intermolecular.
Western blotting was used to detect the higher molecular weight species that form due to intermolecular disulfides between tubulin subunits. Following oxidation by HOSCN, tubulin species (7.5μg per lane, 15μl reactions) were separated by SDS-PAGE under nonreducing conditions. The gels were equilibrated in transfer buffer prior to constructing a gel “sandwich” for transfer to a membrane. Proteins were transferred from the polyacrylamide gels to polyvinylidene difluoride (PVDF) membranes electrophoretically using Owl HEP-1 Semi Dry Electroblotting System (Thermo Scientific) at 10V for 30 minutes.

Nonspecific binding sites on the PVDF membrane were blocked with 3% nonfat milk for 1 hour with shaking. The mouse monoclonal anti-β-tubulin primary antibody was added (7μl antibody in 10mL total), and incubated for 1 hour at room temperature with shaking. The membrane was washed twice for 5 minutes with 10mL of 1X phosphate-buffered saline (PBS) with 0.1% Tween. 1.5μl of the goat anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody in 10mL of PBS-Tween was added to detect the β-tubulin/antibody complex. The membrane was incubated with the secondary antibody for 1 hour at room temperature with shaking, and two washes with 10mL PBS-Tween were done for 5 minutes each. The Pierce West Pico chemiluminescence substrate was prepared, and the membrane was covered with the substrate and allowed to react for 5 minutes at room temperature. The excess substrate solution was removed, and the membrane was wrapped in plastic wrap for imaging. In the darkroom, the membrane was exposed to film, and the film was developed and fixed.
Table 4: Volumes of tubulin, PB pH 7.4, and oxidant used in reactions that were analyzed with Western blot. The concentration of tubulin cysteines in each sample was 100μM and the concentration of oxidant varied from 25-100μM.

Assay for methionine oxidation

To assess the ability of HOSCN to oxidize methionine residues in tubulin, an oxidation reaction was prepared containing of tubulin (5μM, 100μM cysteines, 140μM methionines) and 100μM HOSCN in PB pH 7.4 (total reaction volume of 16μl). Samples were mixed and allowed to react for 20 minutes at room temperature. Samples were acidified with 70% formic acid to a pH of 2.5, and vortex mixed. CNBr was added to achieve a final concentration of 46.5 mM. After overnight incubation with CNBr, samples were neutralized to pH 7.2-7.4 with ammonium hydroxide (NH₄OH), and separated by SDS-PAGE on 7.5% polyacrylamide gels under reducing conditions. Proteins were transferred to PVDF membranes, blocked with 3% milk, treated with mouse anti-β-tubulin for 1 hour, and visualized with the goat anti-mouse HRP conjugate and the chemiluminescence substrate.
**Microtubule polymerization assay**

Purified tubulin, in PME buffer (0.1M Pipes pH 6.9, 1mM MgSO$_4$, 2mM EGTA), was treated with HOSCN in PB pH 6.4 for 10 minutes at 25°C. The final reaction volume was 50μl, the final tubulin concentration was 25μM (500μM cysteine), and HOSCN concentrations were 25μM, 50μM, and 75μM. Reactions were also prepared containing 50μM and 75μM HOCl. To induce the polymerization of tubulin dimers, GTP was added to the samples (final concentration was 1mM), and they were incubated for 22 minutes at 37°C. The polymerized microtubules were collected by centrifugation at 16,000g for 20 minutes, the supernatant was removed, and the protein concentration in the supernatant was determined by BCA protein assay.

**Results and Discussion**

The Landino lab is interested in the effect of cysteine oxidation on the ability of tubulin to form microtubules, but many of the oxidants used in lab oxidize cysteine as well as other amino acids. Therefore, determining the effect of cysteine oxidation alone on tubulin was the goal. HOSCN is a useful chemical tool for probing the effects of cysteine oxidation, but it had never been used previously in Dr. Landino’s lab. The protocol for synthesis of HOSCN had to be developed. It was determined that LPO and SCN$^-$ should be mixed first, and H$_2$O$_2$ should be added to the reaction mixture last for LPO to successfully catalyze the formation of HOSCN. It was also determined that high concentrations of H$_2$O$_2$ and SCN$^-$ retarded LPO activity. Optimal conditions for the formation of HOSCN were found to be 0.645μM LPO, 1mM KSCN, and 0.6mM H$_2$O$_2$ at pH 6.4 to produce approximately 215μM HOSCN.
The tubulin stock solution used for oxidation reactions was 2.65μg/μl, and the GAPDH stock solution used was 2μg/μl. All oxidation reactions were set up to contain 100μM protein cysteines. This was achieved by determining the protein concentration (in molarity) and multiplying it by the number of cysteine residues, 20 for tubulin and 4 for GAPDH. The concentration of protein methionine residues was also determined by multiplying by the number of methionines, 28 for tubulin and 9 for GAPDH. The protein concentrations and the concentrations of protein cysteines and methionines in the stored protein solutions are shown below (Table 5). Appropriate volumes of protein solution were added to reactions to achieve the desired final cysteine concentration of 100μM.

Since GAPDH is a good model protein to use in the laboratory, most experiments were carried out for both GAPDH and tubulin, but only the tubulin results are shown in this paper. The oxidation of GAPDH by HOSCN followed the same patterns as tubulin oxidation. Since the concentration of HOSCN produced was 215μM, we were limited and unable to observe the reaction of protein thiols in an excess of HOSCN. Reactions were typically set up to contain 25, 50, 75, and 100μM HOSCN, or other oxidants.

<table>
<thead>
<tr>
<th>Protein Concentrations</th>
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<tbody>
<tr>
<td>MW (kDa)</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Tubulin</td>
</tr>
<tr>
<td>GAPDH</td>
</tr>
</tbody>
</table>

Table 5: Protein and target amino acid concentrations.

The first goal was to show that HOSCN does in fact oxidize protein thiols. Tubulin samples were oxidized with increasing amounts of HOSCN, and subsequently labeled with the thiol specific labeling reagent IAF. As expected, higher concentrations of HOSCN resulted in less IAF incorporated into the protein, and the protein bands on a
reducing gel got lighter as the concentration of HOSCN was increased (Figure 13). A control lane containing only tubulin was run on the gel, which showed the extent of IAF labeling of tubulin if no oxidant is present (lane 1). Figure 13 shows that HOSCN is oxidizing tubulin cysteine residues. It is also important to note that both the α-tubulin and β-tubulin subunits got lighter as the concentration of oxidant was increased, suggesting that HOSCN does not show specificity for either of the subunits, and both are targets for HOSCN-induced oxidation.

![Figure 13: Oxidation of tubulin cysteines by HOSCN. Lane one contains the control (5μM tubulin), lanes 2-5 contain 5μM tubulin and 25-100μM HOSCN. As the concentration of HOSCN increases, the IAF labeled protein bands get lighter, indicating a greater amount of cysteine oxidation. Both α- and β-tubulin bands are oxidized by HOSCN. Each lane contained 15μg tubulin.](image)

SDS-PAGE of IAF labeled tubulin showed qualitatively that HOSCN oxidizes cysteine residues. To obtain quantitative data, IAF labeled tubulin was precipitated in 80% ethanol and protein pellets were obtained by centrifugation, followed by resuspension in 6M guanidine-HCl. The resuspended protein samples were transferred to a 96 well plate, and fluorescein incorporation was detected at 490nm. The absorption of each sample was compared to the absorption of a control, containing only IAF labeled tubulin, and graphed, showing the percentage of the control reduced cysteines remaining in each sample (Figure 14). It was determined that the addition of HOSCN to tubulin
decreases the number of reduced thiols, and corresponds with what we observed on the IAF labeled gel. When 100μM tubulin cysteine was oxidized with 25μM HOSCN, only approximately 58% of the reduced thiols remained. This shows nearly a stoichiometric relationship, since one molecule of HOSCN is capable of oxidizing two cysteine residues. If the reaction were stoichiometric, only 50% of the reduced thiols would have remained. Treatment with 50, 75, and 100μM HOSCN resulted in approximately 29%, 22%, and 17% reduced cysteines remaining respectively. While these results stray farther from a stoichiometric relationship, it is important to remember that HOSCN decomposes readily and may not be capable of oxidizing all of the cysteine residues in the protein.

Unfortunately, absorption analysis does not allow us to distinguish between α-tubulin and β-tubulin, but the SDS-PAGE results clearly show that both subunits are oxidized. Controls using SDS-PAGE and the plate reader were also performed to confirm that the excess SCN⁻ present does not lead to cysteine oxidation.

*Figure 14: Graph of oxidation of tubulin cysteines by HOSCN. Graphical representation of the SDS-PAGE results in figure 13, obtained from plate reader absorption values. The concentration of tubulin cysteines in each sample was 100μM, treated with 25, 50, 75, or 100μM HOSCN for 20 minutes at room temperature. Samples were labeled with IAF for 30 minutes, precipitated in 80% ethanol, and resuspended in 6M guanidine-HCl. % control values were obtained from the average of 2 experiments.*
The oxidation of tubulin cysteines by HOSCN was compared to the oxidation of cysteines by other oxidants, including HOCl, H₂O₂, and glycine chloramine (GC). Tubulin (5μM, 100μM cysteine), was treated with 100μM of each oxidant, labeled with IAF for 30 minutes, and resolved by SDS-PAGE. Under the experimental conditions, it was determined that HOSCN induced the most cysteine oxidation, when compared to the other oxidants tested (Figure 15). As expected, HOSCN (lane 2) appears to possess a higher specificity for thiols and is a more potent cysteine oxidant than NaOCl (lane 3), H₂O₂ (lane 4), or glycine chloramine (lane 5).

![Figure 15: Oxidation of tubulin cysteines by HOSCN, NaOCl, H₂O₂, and glycine chloramine (GC). Lane one contains the control (5μM tubulin), lanes 2-5 contain 5μM tubulin and 100μM HOSCN, NaOCl, H₂O₂, or GC, respectively. Lane 2 contains the lightest band, indicating that HOSCN induces more cysteine oxidation than the other oxidants. The protein bands in the other lanes, which contain different oxidants, are closer to the brightness of the control band.](image)

Identical samples were also prepared and analyzed for fluorescein incorporation at 490nm as described above. The results obtained from the plate reader supported the results observed by SDS-PAGE. All oxidant concentrations were 100μM, and the percentage of the control reduced cysteines remaining was determined and graphed (Figure 16). According to the absorbance values, 19% of control cysteine residues remained reduced when treated with HOSCN, followed by 63% for NaOCl, 65% for glycine chloramine, and about 90% of the reduced cysteines remained when treated with H₂O₂. The absorbance data support the findings of other researchers that HOSCN has a
higher specificity for thiols than the other oxidants tested. The other oxidants used in this experiment are capable of oxidizing cysteine as well as some other amino acids, such as methionine and tyrosine. While NaOCl, H₂O₂, and glycine chloramine are not as effective as HOSCN at oxidizing cysteine residues, they could be responsible for more overall oxidation involving other amino acids.

![Graph](image)

**Figure 16**: Graph of oxidation of tubulin cysteines by HOSCN, NaOCl, H₂O₂, and glycine chloramine (GC). IAF labeled protein pellets were obtained and resuspended in 6M guanidine-HCl, followed by fluorescein detection at 490nm. HOSCN induces more cysteine oxidation than the other oxidants (19% of reduced cysteines remain). The other oxidants tested oxidize tubulin cysteines to a lesser extent than HOSCN. % control values were obtained from the average of 2 experiments.

To assess the ability of HOSCN to induce the formation of higher molecular weight species through interchain disulfide bonds, oxidized tubulin species were resolved by SDS-PAGE under nonreducing conditions, transferred to a PVDF membrane, and probed with anti-β-tubulin. In figure 17, lane 1 contains the control tubulin sample (5μM, 100μM cysteine) and lanes 2-9 contain tubulin that was oxidized with 25, 33, 42, 50, 58, 67, 75, or 100μM HOSCN. Higher molecular weight dimers and tetramers were detected for all concentrations of HOSCN, but it appeared that at the highest concentrations of
HOSCN (lanes 7-9), fewer dimers and tetramers are formed. The most interchain dimers and tetramers were detected at HOSCN concentrations of 33, 42, and 50μM (lanes 4-6), but this finding is in disagreement with the IAF labeling data, which showed that higher concentrations of HOSCN caused a greater extent of cysteine oxidation.

Since the results do not follow what was expected, we developed two main explanations for why there were fewer dimers and tetramers detected at higher concentrations of HOSCN where there should be more high molecular weight species. The first explanation is that one of the decomposition or reaction products of HOSCN could have the ability to reduce disulfides, while maintaining more total cysteine modifications, thus resulting in a decrease in IAF incorporation. Little is known about the various decomposition products, and this seemed like a plausible explanation. The second explanation is that species larger than tetramers were forming due to disulfides, and they

Figure 17: Detection of interchain tubulin disulfides due to oxidation by HOSCN. Samples were separated by SDS-PAGE under nonreducing conditions, transferred to PVDF, and probed with anti-β-tubulin. Lane 1 is the tubulin control. Tubulin (5μM, 100μM cysteine) was oxidized for 20 minutes at room temperature with 25, 33, 42, 50, 58, 67, 75, or 100μM HOSCN (lanes 2-9). β-tubulin monomers (50kDa), dimers, and tetramers are labeled.
were so large that they remained in the stacking gel, and were never even resolved by SDS-PAGE.

To test the first hypothesis, tubulin was oxidized with ONOO\(^-\) to show the formation of interchain disulfides due to ONOO\(^-\). Tubulin (5μM, 100μM cysteine), was reacted with 25, 50, 75, 100, 125, 150, 175, or 200μM ONOO\(^-\) for 5 minutes, and tubulin species were resolved by SDS-PAGE under nonreducing conditions. ONOO\(^-\) caused interchain disulfides to form, and more dimers and tetramers were detected as the concentration of ONOO\(^-\) increased (Figure 18).

Figure 18: Detection of interchain tubulin disulfides due to oxidation by ONOO\(^-\). Samples were separated by SDS-PAGE under nonreducing conditions, transferred to PVDF, and probed with anti-β-tubulin. Lane 1 is the tubulin control. Tubulin (5μM, 100μM cysteine) was oxidized for 5 minutes at room temperature with 25, 50, 75, 100, 125, 150, 175, or 200μM ONOO\(^-\) (lanes 2-9) in PB pH 7.4. β-tubulin monomers (50kDa), dimers, and tetramers are labeled.

The effect of ONOO\(^-\) alone on tubulin was characterized, and next tubulin was oxidized by ONOO\(^-\) and HOSCN, which had been left to partially decompose for 10 minutes at room temperature, was added to assess the ability of HOSCN decomposition products to reduce interchain disulfides. Tubulin (5μM, 100μM cysteine), was reacted with 200μM ONOO\(^-\) for 5 minutes at room temperature. Next, the HOSCN mixture was added, and the total reaction volume was doubled, so tubulin was now present in 2.5μM.
The results of this experiment were obtained by nonreducing SDS-PAGE, followed by transfer of the protein to a PVDF membrane, and probing for tubulin with anti-β-tubulin. The findings are similar to the results when tubulin was oxidized with only HOSCN (see figure 17), indicating that after 10 minutes of decomposition, HOSCN is still present in high enough concentrations to induce the formation of higher molecular weight disulfides. Additionally, these results suggest that HOSCN decomposition products formed after ten minutes do not reduce interchain disulfides formed by ONOO⁻. It even appears that more of the β-tubulin monomer is oxidized to dimer, and that some of the dimer produced by ONOO⁻ oxidation was further oxidized upon the addition of HOSCN (Figure 19). Western blots of tubulin oxidized by ONOO⁻ followed by treatment with partially decomposed HOSCN resembled the previously shown image of tubulin oxidized with only HOSCN (see figure 17) more than the image showing the effect of only ONOO⁻ on tubulin (see figure 18).

Figure 19: Detection of tubulin oxidized by ONOO⁻ and treated with HOSCN. Tubulin (5μM, 100μM) cysteine was oxidized with 200μM ONOO⁻ for 5 minutes at room temperature. Then the samples were treated with increasing concentrations of HOSCN (new concentration of tubulin is 2.5μM). Lane 1 is the tubulin control. Lane 2 is the ONOO⁻ control, and contains only tubulin and ONOO⁻. Lanes 3-9 contain tubulin, ONOO⁻, and 12.5, 25, 33, 42, 50, 75, or 100μM HOSCN.
To determine whether tubulin was forming molecular weight species larger than tetramers, tubulin was oxidized by ONOO\(^-\) and HOSCN was added as described above. The protein species were resolved by nonreducing SDS-PAGE, and the stacking gel was not cut off prior to transferring the proteins to a membrane. During electrophoretic transfer, the PVDF membrane was positioned to cover the stacking gel, so any protein species remaining in the well would be transferred to the membrane, and could be visualized by Western blotting.

It was determined that while the concentration of ONOO\(^-\) remained constant in each sample, increasing the concentration of HOSCN resulted in more tubulin species remaining in the well (Figure 20). This indicates that dimers and tetramers were being oxidized to even higher molecular weight species, which could not be resolved by gel electrophoresis. It is interesting to note that higher concentrations of HOSCN seemed to preferentially oxidize tubulin dimers and tetramers to even higher multimers before oxidizing more of the monomer to the dimeric form.

![Figure 20: Detection of multimer tubulin species that remained in the wells of the stacking gel. Tubulin (5μM, 100μM) cysteine was oxidized with 200μM ONOO\(^-\) for 5 minutes at room temperature. Then the samples were treated with increasing concentrations of HOSCN (new concentration of tubulin is 2.5μM). Lane 1 is the tubulin control. Lane 2 is the ONOO\(^-\) control, and contains only tubulin and ONOO\(^-\). Lanes 3-9 contain tubulin, ONOO\(^-\), and 12.5, 25, 33, 42, 50, 75, or 100μM HOSCN. Stacking gel was left on during transfer, and higher molecular weight species were detected in the wells of the stacking gel.](image-url)
The ability of HOSCN to oxidize tubulin cysteines has been well characterized, but since the main goal was to characterize the effect of cysteine oxidation only on tubulin, it was necessary to show that HOSCN does not oxidize methionine. To determine whether or not HOSCN is capable of oxidizing methionine, we used a CNBr cleavage assay. Under acidic conditions, cyanogen bromide cleaves peptide bonds at the carboxyl-terminal of reduced methionine residues, and thus is a useful tool for investigating the oxidation of methionine. Cyanogen bromide is not capable of cleaving peptide bonds adjacent to oxidized methionine residues. Therefore, this proteolysis reaction enables us to determine whether or not HOSCN can oxidize the thioether of methionine. If HOSCN oxidized methionine, no tubulin cleavage would occur upon treatment with CNBr. If HOSCN did not oxidize methionine, as we expected, cleavage of tubulin should occur in the same manner as a CNBr control, which was not oxidized prior to CNBr treatment.

To assess methionine oxidation by HOSCN, four controls were needed. The first control contained only tubulin (5μM, figure 21 lane 1). The second control contained tubulin (5μM), formic acid, and ammonium hydroxide (figure 21 lane 2). The third control was a salt control to ensure that the excess SCN⁻ present from producing HOSCN was not interacting with methionine residues or the cleavage reaction (figure 21 lane 3). This control contained tubulin (5μM), SCN⁻ (365μM), formic acid, and ammonium hydroxide. The fourth control was the cyanogen bromide control, and it contained tubulin (5μM), formic acid, cyanogen bromide (46.5mM), and ammonium hydroxide (figure 21 lane 4). The oxidation reaction contained tubulin (5μM) and HOSCN (100μM) which
reacted for 20 minutes, prior to being treated with formic acid, cyanogen bromide, and ammonium hydroxide as described above (figure 21 lane 5).

Comparing lane 2 to the tubulin control in lane 1 indicates that the addition of acid/base causes some protein fragmentation, but this is not significant because it is different from the CNBr control (lane 4). The salt control (lane 3) indicates that SCN⁻, formic acid, and ammonium hydroxide do not cleave tubulin. In figure 21, lane 4 contains the CNBr control, and there is less β-tubulin present when compared to the tubulin control, acid/base control, and the salt control (lanes 1-3, respectively). This indicates that CNBr cleavage successfully occurred. When tubulin is oxidized with HOSCN (100μM) and subsequently treated with CNBr, cleavage occurs similarly to the CNBr control (compare lanes 4 and 5), indicating that methionine residues are not oxidized by HOSCN.

Next, the ability of HOSCN to inhibit tubulin microtubule polymerization was assessed. Since we demonstrated that HOSCN oxidizes only cysteine residues, and not
methionine, we are able to determine the effects of cysteine oxidation only on microtubule polymerization activity. One problem presented was that HOSCN was prepared in PB pH 6.4, but tubulin is stored in PME buffer and polymerization experiments are typically done in PME buffer because Mg$^{2+}$ is required for polymerization. To account for the buffer differences, two sets of samples were prepared and diluted to the final reaction volume, one set with phosphate buffer, and the other with PME buffer. HOSCN inhibition of microtubule polymerization was also compared to inhibition by HOCl. Since tubulin oxidation typically inhibits microtubule polymerization, the supernatant protein concentration should be higher in the samples containing higher concentrations of HOSCN, indicating that less protein polymerized. Control experiments were also performed to ensure that SCN$^-$ alone did not inhibit microtubule polymerization.

HOSCN does inhibit microtubule polymerization in both the PME buffer samples and the phosphate buffer pH 6.4 samples. Additionally, HOSCN inhibits polymerization to a greater extent than equimolar concentrations of HOCl. Even low concentrations of the oxidants (25, 50, 75μM), when compared to the concentration of tubulin cysteine residues (500μM protein cysteines), were capable of inhibiting microtubule polymerization. Table 6 and figure 22 show the effects of HOSCN and HOCl on tubulin polymerization when PB pH 6.4 was used to dilute the oxidation reactions. Table 7 and figure 23 show the effects of HOSCN and HOCl on tubulin polymerization when PME buffer was used to dilute the oxidation reactions.
<table>
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<th>Oxidant (µM)</th>
<th>HOSCN</th>
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<tr>
<td>0</td>
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<td>100</td>
</tr>
<tr>
<td>25</td>
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</tr>
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</tr>
<tr>
<td>75</td>
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Table 6: Concentrations of HOSCN and HOCl used to oxidize tubulin (25µM) prior to initiating microtubule polymerization. These values reflect samples that were oxidized using PB pH 6.4 to dilute to the final volume. HOSCN and HOCl both inhibit polymerization activity, presented as a % of the control tubulin sample.

Figure 22: Effects of HOSCN and HOCl on microtubule polymerization. Tubulin samples (25µM, 500µM cysteine, reaction volume 50µl), were treated with HOSCN or HOCl for 10 minutes, using PB pH 6.4 to dilute the samples. GTP (1mM final) was added to initiate polymerization, and the samples were incubated for 22 minutes at 37°C. Polymerized microtubules were collected by centrifugation at 16,000g for 20 minutes, and the protein concentration in the supernatant was determined by BCA assay.
<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>HOSCN</th>
<th>HOCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>25</td>
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<tr>
<td>75</td>
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Table 7: Concentrations of HOSCN and HOCl used to oxidize tubulin (25μM) prior to initiating microtubule polymerization. These values reflect samples that were oxidized using PME buffer to dilute to the final volume. HOSCN and HOCl both inhibit polymerization activity, presented as a % of the control tubulin sample.

Figure 23: Effects of HOSCN and HOCl on microtubule polymerization. Tubulin samples (25μM, 500μM cysteine, reaction volume 50μl), were treated with HOSCN or HOCl for 10 minutes, using PME buffer to dilute the samples. GTP (1mM final) was added to initiate polymerization, and the samples were incubated for 22 minutes at 37°C. Polymerized microtubules were collected by centrifugation at 16,000g for 20 minutes, and the protein concentration in the supernatant was determined by BCA assay.
Conclusions and future directions

It is believed that the accumulation of oxidative damage plays a significant role in the aging process, as well as inflammatory diseases, heart disease, and neurodegenerative diseases. Cysteine and methionine residues in proteins are the most prone to oxidative attack by a variety of ROS and play an important role in the redox state of cells. Tubulin, a protein that is abundant in neurons, has been found damaged in the brains of patients who suffered from Alzheimer’s disease. The fact that damaged tubulin has been linked to Alzheimer’s disease, coupled with the oxidative stress view of aging, provides motivation to understand the implications of oxidative damage to tubulin on the chemical level.

Dr. Landino’s lab has shown that a variety of oxidizing agents inhibit the ability of tubulin to polymerize and form microtubules. Her lab also determined that the addition of disulfide reducing agents cause tubulin to regain a significant portion of its polymerization activity, suggesting that cysteine oxidation is at least partially responsible for the inhibition of polymerization. All of the oxidizing agents previously tested were not specific for a single amino acid, and they could damage cysteine as well as other amino acids. Therefore, in the present study we used HOSCN, a thiol specific oxidant, to assess the effect of cysteine oxidation alone on tubulin. We found that HOSCN oxidizes cysteine residues in tubulin and induces the formation of higher molecular weight species through interchain disulfide bonds. We also confirmed that HOSCN does not cause methionine oxidation. Although HOSCN is not physiologically relevant in brain tissue where tubulin is abundant, it is a useful chemical tool for assessing the effects of cysteine oxidation on tubulin. Additionally, HOSCN is a physiologically relevant oxidant generated by peroxidase enzymes in tissues other than the brain.
HOSCN inhibits microtubule polymerization to a greater extent than HOCl, likely because it is a thiol specific oxidant unlike HOCl which is capable of reacting with other amino acids, such as methionine. We also demonstrated that HOSCN oxidizes more cysteines than HOCl. Dr. Landino’s lab previously reported that cysteine oxidation, not methionine oxidation, is associated with a loss of polymerization. Therefore, it is reasonable that HOSCN, which oxidizes more cysteines than HOCl, also inhibits polymerization more effectively. Additionally, since cysteine residues are easily reduced and polymerization activity is restored, it is possible that tubulin plays a role in oxidative stress in cells.

Since the system we studied contained only tubulin and HOSCN, it would be interesting to develop a more physiological model in which HOSCN was produced in the presence of tubulin. This could be achieved by incubating LPO, SCN⁻, and H₂O₂ in the presence of tubulin, and then assessing cysteine oxidation of tubulin. Since H₂O₂ would also be present in the solution, it could be another source for cysteine oxidation. It would perhaps be more physiologically relevant to study a system consisting of tubulin and MPO. MPO also catalyzes oxidation of thiocyanate to produce HOSCN, and MPO has been found expressed in the brains of Alzheimer’s patients.

It would also be beneficial to develop experimental conditions to produce a higher concentration of HOSCN. Many papers report that LPO catalyzed production of HOSCN results in concentrations in the millimolar range, but we were only able to achieve approximately 0.215mM. A higher concentration of HOSCN would allow us to explore a wider range of the effects of HOSCN-mediated oxidation.
Additionally, the effect of oxidants on tubulin *in vivo* could be assessed using the yeast *S. cerevisiae* as a model organism. Yeast cells could be treated with various oxidants, and cell lysates could be analyzed for tubulin cysteine oxidation. The tubulin content from yeast cells treated with oxidants could be compared to control cells which were not oxidized. The proteins could be labeled with IAF, and resolved by SDS-PAGE. The bands on the gel would get lighter if tubulin is oxidized, since there would be less IAF incorporation. Additionally, we could determine if tubulin interchain disulfides are formed by Western blot. *In vivo* yeast studies would be an exciting step for Dr. Landino’s laboratory, and could provide interesting insight on how oxidants *truly* interact with microtubules in a biological system.
Acknowledgements

Firstly, I would like to thank Dr. Lisa Landino for being my advisor. I truly enjoyed working as a research student in her lab, and I doubt a better research advisor exists. Dr. Landino provided invaluable knowledge and guidance to me throughout the last year and a half. She was always available to offer help and encouragement when I struggled with my honors project, while also giving me the independence to grow and think as a researcher. I learned an incredible amount of chemistry from working in Dr. Landino’s lab, but more importantly, I learned how to think about a problem and develop approaches to solve it. Dr. Landino inspired me to work harder and learn more, and I will always be grateful for the time I spent conducting research in her lab.

I would like to thank Dr. Gary Rice and Dr. Lizabeth Allison for serving on my committee. Thank you for taking the time to read my thesis and attend my defense.

Additionally, I need to thank the other members of Dr. Landino’s lab—Tara Hagedorn and Stephanie Brooks. They taught me so much in lab, and kept me laughing the whole time.

I would also like to thank my parents, grandparents, Brad, Alex, and Adam. Their encouragement and support during my time at William and Mary helped me a tremendous amount. I wouldn’t have been able to do this without their support. Thank you to my parents and grandparents for instilling a sense of scientific curiosity in me at an early age.
References


