Inhibition of Oxidation-Induced Misfolding of Mutant SOD1 in ALS using Small Molecules

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Biochemistry
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2016

Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the loss of motor neurons resulting in progressive paralysis that eventually leads to the death of an afflicted individual. In a majority of ALS cases, the cause of onset is unknown, but a small subset of ALS cases are linked to mutations in the protein Cu, Zn superoxide dismutase (SOD1). Despite extensive research in this area, few treatments for ALS have been developed. Since the description of the disease by Jean-Martin Charcot in the mid-nineteenth century, only one drug, Riluzole has been approved in the management of this disease.

We propose investigating therapeutic treatments of ALS by looking for small molecules that have the potential to stabilize the native structure of mutant SOD1 and prevent its misfolding and aggregation. We started our investigation by first devising a method to optimize the purification of wildtype and mutant SOD1 in our lab. We constructed a fusion protein of SOD1 linked to a yellow fluorescent protein, a hexahistidine tag, and a tobacco etch virus (TEV) cleavage site and showed that both wildtype and mutant SOD1 purified from this construct was active and metallated.
We used our purified protein to explore the results of an *in silico* binding screen searching for SOD1 native-state binders. We discovered the flavonoid, epigallocatecin-3-gallate (EGCG) was able to inhibit the misfolding and aggregation of the ALS-causing mutation A4V-SOD1 exposed to oxidative stress. Additionally, we have evidence that the inhibitory effect of EGCG occurs through the stabilization of the dimer interface of mutant SOD1, rather than the anti-oxidant properties of this molecule. This is the first report of EGCG having anti-aggregation properties in an ALS model and these results implicate the potential therapeutic value of EGCG within the field of SOD1-ALS.
Acknowledgments

The path to obtaining my PhD has encompassed many ups and downs, but I consider myself very fortunate to have a supervisor as understanding and supportive as Avi Chakrabartty. He has been that stern enforcer when I needed to regain focus, a compassionate individual during my downs, and an encouraging teacher throughout it all. I have had a wonderful experience in working in his lab and I have truly learned so much from him regarding research and life. I would also like to thank my supervisory committee members, Angus McQuibban and Bill Trimble. You have been the absolute best and I feel lucky to have two of the nicest and most encouraging individuals on my committee. You always made me feel like you were on my side and working with me to achieve my goals. I thank you so much for your input, kindness, and support throughout my time at the University of Toronto.

I would like to thank all my lab mates on the forth floor, past and present, who have not only taught me so much over the years, but have become really good friends. A special thanks to Rishi Rakhit, who left the lab, but could not get SOD1 out of his head and had a friend run an in silico drug screen that reignited my failing project.

Lastly, I would like to thank my family for all their love and support throughout my life. I especially thank my mom who raised me with the confidence to be able to achieve and my husband for providing the support that allowed me to continue when things became difficult.
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Chapter One

Introduction

Summary

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that is caused by the death of motor neurons in the motor cortex, brainstem, and spinal cord of patients. This disease involves progressive muscle loss leading to paralysis, which eventually becomes fatal. Here, I review the clinical features of ALS and describe many of the known genetic causes of the disease with particular focus on mutations of Cu, Zn superoxide dismutase (SOD1). Various theories regarding the toxic mechanism of ALS are summarized and finally therapeutic strategies within the field are discussed. Overall, it is clear that ALS is a multifaceted disease with complicated features and while much progress has been made in understanding this disease, there is still a great deal that needs to be discovered regarding its cause and toxic mechanism. In achieving improved understanding of ALS, the development of better therapeutics can eventually be realized.

ABBREVIATIONS: ALS – Amyotrophic lateral sclerosis, MND – Motor Neuron Disease, FTLD - frontotemporal lobe degeneration, TDP-43 - TAR DNA-binding protein 43, SOD1 - Cu, Zn superoxide dismutase, FUS – Fused in sarcoma, CCS – Copper chaperone for SOD1
The Disease of Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a late-onset neurodegenerative disease that was first characterized by Jean-Martin Charcot in the mid-nineteenth century.\textsuperscript{1,2} Charcot described ALS patients as having symptoms that included muscle weakness progressing to paralysis, spasticity or rigidity of limbs, difficulties in swallowing, and respiratory failure.\textsuperscript{2,3} He also described patients preservation of cognitive function as well as the sustainment of eye movement, bladder, and bowel control.\textsuperscript{2,3} Today, this description of the disease remains mostly intact and it is referred to as classical ALS or Charcot ALS. In these cases of ALS, the occurrence of deterioration of both upper and lower motor neurons is present, lower motor neuron degeneration corresponds to muscle weakness and atrophy and upper motor neuron degeneration includes slowing of movement, hyper-reflexia, and spasticity.\textsuperscript{4}

The classification of ALS becomes complicated for many reasons. One instance is the presence of ALS cases that demonstrate only lower motor neuron degeneration referred to as pure lower motor neuron type progressive muscle atrophy or strictly upper motor neuron degeneration referred to as pure upper motor neuron type primary lateral sclerosis.\textsuperscript{5} A further complication is that each of the pure upper or lower motor neuron ALS cases can eventually develop deterioration in motor neurons of the opposite region in the later stages of the disease.

Another atypical form of ALS is referred to as predominant bulbar palsy, which is identified by symptoms localized to the bulbar region for a prolonged period (over 6 months). Symptoms usually include dysphagia, dysarthria, hoarsness, and tongue wasting.\textsuperscript{6} Bulbar-onset ALS is found in approximately 20\% of ALS cases.\textsuperscript{6}

In addition to the atypical forms of ALS, another complication in classifying the disease is ALS belongs to a broader family of diseases referred to as motor neuron diseases (MNDs). Only recently has it been discovered that overlap exists between many of the MNDs and other
neurodegenerative diseases that were once considered distinct. For instance, in the later stages of ALS, about 50% of patients present with some cognitive damage, this causes overlap with another disease, frontotemporal lobe degeneration (FTLD), which is characterized by progressive impairment of behaviour and language.\textsuperscript{7,8} Interestingly, both diseases demonstrate the presence of inclusions containing TAR DNA-binding protein 43 (TDP-43)\textsuperscript{9} and mutations in chromosome 9 open reading frame 72 (C9ORF72) have been linked to both ALS and FTLD.\textsuperscript{10} This considerable overlap suggests some connection between the two diseases. Therefore, it is proposed that pure ALS as described by Charcot is considered an extreme end on a spectrum of diseases (Fig.1) and as atypical symptoms arise, diagnosis of the disease may change to incorporate these features.\textsuperscript{11}

FIGURE 1: ALS as a spectrum disease. Pure ALS and pure FTLD are two distinct neurodegenerative diseases that affect different areas of the brain and present with different symptoms. Patients can sometimes demonstrate features from both diseases, which complicates diagnosis and leads to the classification of ALS as a spectrum disease with deviation from classical symptoms first reported by Charcot.

In the 1990’s the incidence rate was determined on average to be 1.89 cases per 100,000 individuals per year whereas the prevalence was 5.2 cases per 100,000 individuals within Western countries.\textsuperscript{12} The mean age of onset is between 55-65 years of age and only 5% of cases have an onset before the age of 30 years old. There appears to be a slight male bias of the disease with a male to female ratio of 1.5:1 being reported in the early nineties.\textsuperscript{12} The typical survival time from symptom onset is approximately 3 to 5 years and mortality rates from the 1990s report
1.54 to 2.55 deaths per 100,000 individuals per year.\textsuperscript{12} The aforementioned data refers to ALS cases that have an unknown cause or simply referred to as sporadic ALS and they account for 90\% of all ALS cases. The remaining 10\% of the disease can be attributed to the autosomal dominant inheritance of a genetic factor and these occurrences are referred to as familial ALS (fALS).\textsuperscript{13}

\textit{Genetic Causes of ALS}

There are slight variations existing between fALS and sALS cases, which include fALS patients having a decade earlier age of onset, effecting both males and females equally, and shorter survival times.\textsuperscript{14,15} The largest cause of fALS, which contributes to over 40\% of cases, is a dominantly inherited hexanucleotide repeat expansion, (GGGGCC)\textsubscript{n}, in the first intron region of the \textit{C9ORF72} gene.\textsuperscript{10,16} Also, this hexanucleotide repeat was detected in approximately 20\% of all sALS cases.\textsuperscript{10,16} The \textit{C9ORF72} gene encodes two protein isoforms, C9ORF72a and C9ORF72b, to date the function of these proteins remains unknown.

Additionally, little is known regarding the mechanism that associates the gene expansion with neurodegeneration. It has been speculated that due to the location of the hexanucleotide repeat in the regulatory region of the gene, that gene expression may be disrupted and in fact, a loss of transcription of this gene has been demonstrated.\textsuperscript{10,16,17} An alternative mechanism regarding C9ORF72 and its involvement in ALS is RNA-mediated toxicity.\textsuperscript{10} This theory is based on observations of the presence of C9ORF72 hexanucleotide repeats in intranuclear RNA foci.\textsuperscript{10} This hypothesis is further supported by a study demonstrating RNA-binding proteins were associated with the C9ORF72 expansion in intranuclear and cytoplasmic inclusions found in brain tissue samples of patients with the expansion repeat.\textsuperscript{18} Recently, a study using pluripotent stem cell differentiated neurons from patients with the C9ORF72 hexanucleotide repeat shed more support to the RNA toxicity hypothesis by demonstrating the sequestering of the RNA
binding protein, ADARB2, with the expansion repeat in RNA foci.\textsuperscript{19} A final hypothesis relating the hexanucleotide repeat of C9ORF72 and its involvement in ALS points to an atypical mechanism of translation, the repeat associated non-ATG translation or RAN-translation. The structure of the hexanucleotide repeat allows for this atypical translation to occur and moreover, the presence of RAN translated insoluble dipeptides was detected through the use of antibodies in nuclear and cytoplasmic inclusions of C9ORF72 ALS patients.\textsuperscript{20} Overall, the various mechanisms regarding the expansion repeat may not be mutually exclusive and they may all contribute to the disease pathology of ALS.

In addition to the hexanucleotide repeat of C9ORF72, mutations in a number of proteins are linked to ALS. For instance, TAR DNA binding protein (TDP-43) and fused in sarcoma (FUS) encode for DNA/RNA processing proteins with functions that include DNA repair, regulation of RNA transport, translation, splicing, and the formation of stress granules.\textsuperscript{21} It has been found that mutations in these two proteins are associated with approximately 6\% of fALS cases as well as up to 2\% of sALS occurrences.\textsuperscript{22–24} Similar to C9ORF72, the elucidation of how mutations in these genes lead to ALS is not well understood, however evidence suggests that multiple mechanisms may participate, which include; a gain of toxic function, a loss of nuclear function, and the appearance of stress granules.\textsuperscript{21}

There are approximately 50 mutations of TDP-43 at its glycine-rich terminal that can lead to ALS.\textsuperscript{22} Increasing the expression of mutant TDP-43 displays an increase in neurodegeneration in transgenic mouse models and the severity of neuronal degeneration is directly linked to protein expression levels, both of which suggests a mechanism that acts through a gain of toxic function.\textsuperscript{25} Additionally, ALS patients show an accumulation of TDP-43 in cytoplasmic aggregates, which results in a loss of nuclear TDP-43 and implies a deficiency of the nuclear function as a toxic mechanism in motor neuron degeneration.\textsuperscript{26} Mutations in FUS occur at the e-
terminal in the nuclear localization domain of the protein and similar to TDP-43, mutant FUS localizes to cytoplasmic inclusions in ALS patients, which again suggests a possible loss of nuclear function. Lastly, the increased association of mutant TDP-43 and FUS with cytoplasmic stress granules is speculated to lead to neuronal degeneration through the sequestering of RNA-binding proteins, which may cause the prevention of RNA translation and the promotion of inclusions. In addition to TDP-43 and FUS, there are other RNA-binding/processing proteins that contribute to fALS. For instance, mutations in angiogenin and senataxin have both been connected to fALS.

In addition to the DNA/RNA processing proteins, other causes of fALS have been found in mutations of proteostatic proteins (eg. Ubiquilin 2), cytoskeleton and cellular transporter proteins (eg. Vesicle-associated membrane protein-associated protein B and C, Peripherin, Dynactin 1, and neurofilament heavy chain), and proteins of unknown function (eg. Alsin or ALS2). The various genetic causes of ALS have been summarized in Table 1. Of all the genetic causes of ALS, the oldest and most studied, involve mutations in the enzyme Cu, Zn superoxide dismutase (SOD1).
Table 1: Genetic causes of ALS\textsuperscript{11}

<table>
<thead>
<tr>
<th>Mutant molecule</th>
<th>Gene locus</th>
<th>Estimate % of fALS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzyme</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superoxide dismutase 1 (SOD1)</td>
<td>21q22.1</td>
<td>20%</td>
</tr>
<tr>
<td>RNA-binding and/or processing protein dysfunctions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAR DNA-binding protein 43 (TDP43)</td>
<td>1p36.2</td>
<td>1–5%</td>
</tr>
<tr>
<td>FUS</td>
<td>16p11.2</td>
<td>1–5%</td>
</tr>
<tr>
<td>TATA-binding protein associated factor 15 (TAF15)</td>
<td>17q11.1–q11.2</td>
<td>Unknown</td>
</tr>
<tr>
<td>Ewing sarcoma breakpoint region 1 (EWSR1)</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Angiogenin (ANG)</td>
<td>14q11.2</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Senataxin (SETX)</td>
<td>9q34</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>Repeat expansions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromosome 9 open reading frame 72 (C9ORF72)</td>
<td>9p21.3–p13.3</td>
<td>40–50%</td>
</tr>
<tr>
<td>Ataxin 2 (ATXN2)</td>
<td>12q24</td>
<td>&lt;1%</td>
</tr>
<tr>
<td><strong>Proteostatic proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ubiquilin 2 (UBQLN2)</td>
<td>Xp11</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Optineurin (OPTN)</td>
<td>10p15–p14</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Sequestosome (SQSTM1)</td>
<td>5q35</td>
<td>Unknown</td>
</tr>
<tr>
<td>Valosin-containing protein (VCP)</td>
<td>9p13</td>
<td>&lt;1%</td>
</tr>
<tr>
<td><strong>Excitotoxicity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-amino-acid oxidase (DAO)</td>
<td>12q24</td>
<td>&lt;1%</td>
</tr>
<tr>
<td><strong>Cytoskeleton/cellular transport deficits</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vesicle-associated membrane protein-associated protein B and C (VAPB)</td>
<td>20q13.3</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Peripherin</td>
<td>12q13.12</td>
<td>Unknown</td>
</tr>
<tr>
<td>Dynactin 1 (DCTN1)</td>
<td>2p13</td>
<td>Unknown</td>
</tr>
<tr>
<td>Neurofilament heavy chain (NFH)</td>
<td>22q12.2</td>
<td>Unknown</td>
</tr>
<tr>
<td>Profilin 1 (PFN1)</td>
<td>17p13.2</td>
<td>Unknown</td>
</tr>
<tr>
<td>Charged multivesicular body protein 2b (CHMP2B)</td>
<td>3p11</td>
<td>Unknown</td>
</tr>
<tr>
<td>Phosphatidylinositol 3,5-bisphosphate 5-phosphatase (encoded by FIG4)</td>
<td>6q21</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>Uncertain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spatacsin</td>
<td>15q21.1</td>
<td>Unknown</td>
</tr>
<tr>
<td>Alsin</td>
<td>2q33.2</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>?</td>
<td>18q21</td>
<td>Unknown</td>
</tr>
<tr>
<td>?</td>
<td>20p tel–p13</td>
<td>Unknown</td>
</tr>
<tr>
<td>?</td>
<td>15q15.1–q21.1</td>
<td>Unknown</td>
</tr>
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\textit{Cu, Zn Superoxide Dismutase}

Eukaryotic SOD1 is composed of 153-amino acid residues that form a dimer complex (Fig.2). The dimer is held together by hydrophobic interactions and four hydrogen bonds and approximately 550 Å of hydrophobic surface area (9% of the external surface) is buried at the interface of the two monomers.\textsuperscript{30} Hence, SOD1 dimerization leads to an increase in protein stability by reducing its solvent accessible surface area. Each monomeric subunit contains eight anti-parallel β-sheet strands that form a β-barrel and the hydrogen bonds between the strands compose a “Greek-key” fold.\textsuperscript{31} Strands 1, 2, 3, and 6 are oriented opposite of the active site and
demonstrate little twisting, whereas strands 4, 5, 7, and 8 are shorter and more twisted.\textsuperscript{32} Also located in the \(\beta\)-barrel of SOD1 are a number of beta bulges, which serve the purpose of accommodating the metal binding capacity of this protein.\textsuperscript{30} Each subunit of SOD1 coordinates one copper atom and one zinc atom that are located in the metal-binding loop of the protein (residues 49-84).\textsuperscript{33} The metal binding of SOD1, along with the presence of an intramolecular disulfide bond between cysteine 57 and cysteine 146 contribute to SOD1’s extraordinary stability.\textsuperscript{30,34,35} Fully metallated SOD1 has a melting temperature of 85-95\(^\circ\)C (depending on buffer conditions)\textsuperscript{36} and it is enzymatically active in 8M urea or 4M guanidine-HCl.\textsuperscript{37}

![Figure 2: Ribbon diagram of SOD1 dimer based on the Protein Data Bank ID 1PU0 and rendered using PyMOL 0.99. The structural representation depicts the eight-stranded \(\beta\)-barrel, copper ion (orange), and zinc ion (grey) found on each monomer.](image)

SOD1 is a ubiquitously expressed free radical scavenger that catalyzes the dismutase reaction that converts two superoxide anions to one molecule of hydrogen peroxide and one molecule of oxygen (Fig.3).\textsuperscript{39} Therefore, it protects the cell against the highly reactive superoxide anion that gets generated as a normal by-product of cellular respiration.\textsuperscript{40} The catalytic rate constant of SOD1 is \(2\times10^9\, \text{M}^{-1}\text{s}^{-1}\), which is believed to be diffusion-controlled based on comparisons with the rate of diffusion of superoxide with SOD1.\textsuperscript{41} Furthermore, driving the
diffusion of the negatively charged superoxide anion is its attraction to the positively charged active site of SOD1. Approximately, 11% of the exposed surface area of SOD1 is attributed to the positively-charged active site, while the remaining surface area is negatively charged, thus the superoxide anion is electrostatically guided towards the active site.\textsuperscript{41,42}

\textbf{FIGURE 3:} The catalytic activity of SOD1. Two molecules of superoxide anion are converted to one molecule of oxygen and one molecule of hydrogen peroxide using the redox reaction of copper.

Once at the active site, the superoxide anion displaces a water molecule to form a hydrogen bond with the arginine 143 amino acid residue. This residue along with threonine 137 helps determine the size of molecule that can enter the active site.\textsuperscript{43} The superoxide anion directly binds to oxidized copper (Cu(II)), which is coordinated to histidines 46, 48, 63, and 120 in a distorted tetrahedral binding geometry (Fig.4), and transfers its electrons.\textsuperscript{43} This converts superoxide to dioxygen, which is no longer a charged molecule and hence it loses its affinity to arginine 143 and diffuses out of the active site. A water molecule binds again to arginine 143 and the copper, in its reduced state becomes trigonal bound to histidine 46, 48, and 120.\textsuperscript{43} The histidine 63 amino acid residue is referred to as the bridging histidine because it binds to both the zinc and oxidized copper (Fig.4). When copper becomes reduced (Cu(I)), histidine 63 moves away from the copper towards the acidic environment of the zinc, where it becomes protonated.\textsuperscript{43} When a second superoxide molecule diffuses into the active site, it again displaces the water
molecule at arginine 143 to form a hydrogen bond with both the amino acid as well as a second water molecule. This superoxide molecule accepts an electron from Cu(I) through a non-bond electron transfer and additionally accepts a proton from the hydrogen-bonded water molecule and a second proton from histidine 63 to form hydrogen peroxide.\textsuperscript{43} The neutral hydrogen peroxide diffuses out of the active site and Cu(II) reforms its bond with the deprotonated histidine 63. Lastly, a chain of water molecules replenishes the protons that were used to form hydrogen peroxide.\textsuperscript{43}

FIGURE 4: A cartoon representation of the coordination of metals in SOD1.\textsuperscript{30,44,45} The histidine 63 amino acid residue binds to both the zinc ion and the oxidized copper ion. The reduction of copper causes histidine 63 to move towards the zinc, which leaves reduced copper coordinated to the remaining three histidines in a trigonal planar geometry (not shown).

While copper is catalytically important to SOD1, the zinc ion helps maintain the structure and stability of the protein. The zinc ion is bound near the active site of SOD1 in a distorted tetrahedral geometry to histidine 63, 71, 80 and aspartate 83 (Fig.4).\textsuperscript{42} The mechanism by which zinc is inserted into SOD1 is unknown, but metallothioneins are thought to be involved.\textsuperscript{46} It has
been shown that metallothionein levels were upregulated in SOD1-ALS mutant mice and ALS victims.\textsuperscript{47,48} Additionally, when transgenic mice carrying an ALS-causing mutation were crossed with mice that had disrupted metallothionein genes, the resulting mice experienced an accelerated onset of ALS and shorter survival times, which may be indicative of early protein structure destabilization and aggregation.\textsuperscript{49}

Alternatively, copper insertion into SOD1 occurs through the copper chaperone for SOD1 (CCS).\textsuperscript{50} The CCS protein is composed of three domains, domain I and III are necessary for copper insertion function, while domain II is homologous to SOD1.\textsuperscript{51} Therefore, domain II can form either a heterodimer or the more energetically favourable heterotetramer with SOD1 in order to insert a copper ion.\textsuperscript{52,53} Exactly how CCS obtains its copper is unknown, but it is speculated that it involves the copper transporter, Ctr.\textsuperscript{53} Another mechanism for copper insertion found only in mammals involves reduced glutathione. The role of glutathione in copper homeostasis is well established and it has been demonstrated that copper in mammalian cells first appears complexed by glutathione before emerging in other copper containing molecules, like SOD1.\textsuperscript{54} Therefore, it is possible that a copper-glutathione complex can act as a copper donor for SOD1, however the possibility that glutathione provides the reducing equivalent for another molecule involved in copper insertion of SOD1 cannot be ruled out.\textsuperscript{55}

\textit{Mutations of SOD1}

In 1993 Rosen et al. published the first account of mutations in the \textit{sod1} gene being linked to ALS.\textsuperscript{56} Since this discovery, over 170 mutations of SOD1 have been shown to cause classic ALS (ALSoD mutation database, accessed on 1\textsuperscript{st} September 2014). The majority of SOD1 mutations are missense mutations scattered throughout the sequence of the protein (Fig.5), however there are a few mutations that result in frameshifts, truncations, deletions, and insertions.\textsuperscript{57} Additionally, no null mutation of SOD1 has ever been linked to ALS and truncated
mutations usually occur near the end of the protein (e.g., G141X), hence it is believed that the SOD1 polypeptide is necessary for cytotoxicity.\textsuperscript{57}

The vast majority of mutations of SOD1 are dominantly inherited, however, the exception is a D90A mutation that is found in Scandinavian populations, which is both dominantly and recessively inherited.\textsuperscript{58} It is postulated that the carriers of the recessively inherited D90A mutation share a common ancestor and the examination of multiple recessive pedigrees demonstrates that not only is this true, but additionally a small region across the SOD1 gene has avoided recombination and is common to all recessive pedigrees.\textsuperscript{59,60} This common region is believed to fall in the regulatory region of SOD1 and it is proposed that a cis-acting polymorphism in this region reduces the transcription of D90A SOD1, hence two copies of the mutation are required for disease expression in individuals carrying the recessive haplotype.\textsuperscript{59}

Overall, mutations of SOD1 are responsible for about 20\% of fALS cases and approximately 2\% of sALS cases, however it is still not fully understood as to how SOD1 mutations result in motor neuron degeneration and ALS.\textsuperscript{56}
FIGURE 5: ALS-causing mutations of SOD1. Over 170 different mutations of SOD1 scattered throughout the sequence of the protein have been shown to lead to ALS, listed are the missense and truncation mutations.

There are many difficulties in the investigation of the mechanism by which mutant SOD1 causes ALS. One major hurdle is that numerous mutations can lead to the disease, but not all mutant SOD1 behave the same. For instance, observing just disease progression, some mutations like the D90A recessive mutation and the H46R mutation show a slow progression of ALS, while the most common mutation found in North American patients, A4V, demonstrates a rapid progression of the disease. Furthermore, there is considerable variation relating to age of onset, clinical features, and disease progression within one ALS-causing SOD1 mutation. For example, one documented family carrying the I113T mutation had one member develop ALS as early as 39 years of age and maintain locomotion five years after diagnosis, while another member who carries the same mutation is 86 years old, but as of yet has not developed any symptoms. The cause of this phenotypic variability amongst patients carrying identical mutations of SOD1 is
likely due to differences in environment and genetic background. It has been shown that mice of different genetic backgrounds carrying a G86R-SOD1 mutation demonstrated differences in disease course. The FVB/N mice developed an ALS phenotype within 100 days, however, when these mice were bred into mice of the mixed background C57B16/129Sv, ALS symptoms did not develop until 140 days to as late as 2 years.\textsuperscript{62} In addition to differences in genetic background, environmental conditions like accumulated amounts of oxidative stress or cellular capability to handle varying amounts of unfolded protein may participate in the presentation of these phenotypic differences.\textsuperscript{63}

One mechanistic feature that is common to all SOD1 mutations is they somehow cause ALS through a gain of toxic function rather than a loss of activity. In support of this, it has been shown that many ALS-causing mutations of SOD1 have high dismutase activity.\textsuperscript{64} Furthermore, studies with transgenic mice expressing mutant human SOD1 alongside of normal endogenous mouse SOD1 show development of ALS, and knocking out the \textit{sod1} gene in mice demonstrated no ALS progression.\textsuperscript{65} To date, this gain of toxic function remains elusive, however the presence of aggregates in motor neurons is a hallmark of the disease that is found in all ALS cases and indicates that protein misfolding is likely involved.\textsuperscript{66}

A number of hypotheses have attempted to explain this gain of toxic function. One theory suggests aberrant enzymatic activity of mutant SOD1 leads to the production of reactive oxygen species that can cause oxidative damage to SOD1 and other cellular protein. In support of this theory, it was shown through monitoring the change in electron spin resonance that mutant A4V-SOD1 and G93A-SOD1 catalyzed the oxidation of the substrate spin trap 5,5’-dimethyl-1-pyrroline N-oxide (DMPO) at a higher rate than wildtype SOD1.\textsuperscript{67} The difference in rate is due to a difference in the binding affinity of mutant SOD1 for hydrogen peroxide.\textsuperscript{68,69} The increased affinity of hydrogen peroxide at the active site of mutant SOD1 leads to the copper ion catalyzing
the reverse dismutase reaction and increasing the production of free radicals, which subsequently causes higher amounts of oxidative damage in the cell. Additionally, other groups have found that mutant SOD1 can bind to several other molecules at its active site leading to the production of other harmful free radical species like peroxynitrite and the carbonate anion radical.

This hypothesis is based on the ability of the copper of SOD1 to catalyze aberrant reactions that increase oxidative stress in the cell environment, however this theory has come into question based on two studies. The first study demonstrated that mice lacking the copper chaperone for SOD1 (CCS) crossed with mice expressing mutant SOD1 did show a decrease in copper loading in SOD1 and while this led to decreased dismutase activity in these mice, it did not alter the severity or survival of the double mutant mouse. The second study showed mice that were transgenically expressing a mutant form of SOD1 in which all four of the histidines responsible for copper coordination were changed to alanines had no change in disease course despite the absence of copper. These studies do not completely rule out the involvement of aberrant copper chemistry in SOD1-ALS pathogenesis. It is possible that poor copper loading in the protein does not necessitate the need of increased free radical production and oxidative damage to produce the toxic, misfolded SOD1 intermediate. This would suggest that an increase in the propensity of SOD1 to misfold is again central to this disease.

Therefore, the most apparent explanation for the shared gain of toxic function of mutant SOD1 is they demonstrate reduced stability of their native structure, which leads to misfolding and aggregation. However, several crystallographic studies showed that most ALS-causing mutations of SOD1 cause very little modification to the SOD1 native structure, and only mutations in the metal binding region of the protein showed some local disorder, while the β-barrel core of these mutations still remained intact. The reduced affinity for metals is not exclusive to mutations in the metal binding region and it is another shared property of many
mutations of SOD1. SOD1 mutations scattered across the protein can lead to changes in the protein’s zinc binding geometry, which can cause greater flexibility in the active site and metal loss. The metal binding affinity of SOD1 is measured indirectly by comparing metal release rates under mild denaturing conditions and examining the competition for these metals with chelators that have a known binding affinity. Using this method, the dissociation constants of zinc and copper of wildtype SOD1 were calculated to be $4.2 \times 10^{-14}$ M and $6.0 \times 10^{-18}$ M respectively and mutant SOD1 had weaker binding affinities for both metals, approximately 20-30 times less for zinc and 1-2 times lower for copper. SOD1 mutations that cannot bind metals have a melting temperature 4-12°C lower than wildtype apo-SOD1, while mutants that can bind metals melt at 1-6°C lower than wildtype holo-SOD1. Despite the slightly lower thermostability of mutant SOD1, the fact remains that this protein is still considered extremely stable with a high melting temperature. This leads to another perplexing problem, how does a protein with high stability misfold and aggregate.

**Oxidation Hypothesis**

One possible explanation of how a stable protein misfolds to cause aggregation is the presence of a denaturant. Our laboratory and others speculate that oxidative stress is the destabilizing mechanism enabling mutant SOD1 to misfold and aggregate. We postulate that SOD1’s role as a free radical scavenger puts it at risk of oxidative damage, which can lead to misfolding primed by the presence of a mutation. This hypothesis puts forth the notion that the shared gain of toxic function of mutant SOD1 is not decreased stability, but instead an increased susceptibility to form an aggregate-prone, native-like misfolded species in response to a denaturing stress.

In support of this hypothesis, our laboratory has identified covalent modifications to active site amino acids under oxidizing conditions and we have demonstrated that these
modifications in mutant SOD1 can cause misfolding and aggregation, while wildtype SOD1 remained fairly aggregate-resistant.\textsuperscript{87–89} Furthermore, we have identified various destabilized SOD1 intermediates that occur in response to oxidation induced by hydrogen peroxide and we have shown that mutant SOD1 was found to misfold more dramatically than wildtype SOD1 in response to this denaturing stress.\textsuperscript{89} In addition to our \textit{in vitro} studies, others have demonstrated that the levels of oxidative stress are elevated in ALS patient tissues\textsuperscript{90–92} and oxidative modifications to proteins, including SOD1, have been identified in transgenic mouse models of ALS.\textsuperscript{93,94}

Overall, the oxidation hypothesis is appealing because it explains many features of ALS. First, the specificity of the disease for the degradation of motor neurons can be rationalized by the view that motor neurons are some of the longest cells in the body, with axons that reach 1m in length.\textsuperscript{88} The transport of SOD1 from the cell body to the end of the axons occurs using the slow component b of the anterograde axonal transport system, which requires the half-life of SOD1 to be hundreds of days.\textsuperscript{88} This long half-life would allow oxidative damage of proteins to accumulate, which in the case of SOD1 could potentially lead to the formation of cytotoxic SOD1 intermediates. Other likely reasons for motor neuron susceptibility in ALS are the high concentrations of SOD1 in these cells\textsuperscript{95} and the high metabolic activity that is required to maintain the sodium-potassium gradient, both of which promote aggregation.\textsuperscript{96} Additionally, the higher metabolic levels required by motor neurons would result in higher mitochondrial activity and increased production of free radical species, like superoxide anion, which encourages more oxidative damage. The oxidation hypothesis also provides an explanation for the adult onset of ALS. Although SOD1 mutations are found in patients from birth, onset of the disease does not usually occur until later in life. This delayed onset may be a result of increased oxidative stress.
occurring with increased age and it is responsible for the misfolding and aggregation of mutant
SOD1.\textsuperscript{96}

\textit{Other Toxic Mechanisms of ALS}

There are a number of alternative hypotheses that attempt to explain the toxic mechanism
of ALS. In addition to the oxidation hypothesis, there is supporting evidence to suggest
 glutamate-mediated excitotoxicity could be a contributing factor in ALS. One suggested
 mechanism of excitotoxicity is the over stimulation of motor neurons by glutamate, leading to an
 influx of calcium ions into the cell, which can upset the calcium homeostasis as well as lead to
calcium overload of the mitochondria.\textsuperscript{97} This overload can cause increased production of reactive
 oxygen species and increased levels of oxidative stress.\textsuperscript{98,99} It has also been suggested that the
 presence of mutant SOD1 in the mitochondria could render motor neurons more sensitive to
 glutamate excitotoxicity, therefore suggesting that the gain of toxic function of mutant SOD1 is
increased sensitivity of motor neurons to glutamate.\textsuperscript{100,101} In addition to excitotoxicity as a
mechanism leading to ALS, it has also been suggested that glutamate sensitivity could be
responsible for motor neuron vulnerability in ALS. Motor neurons display low calcium buffering
capacity due to low expression of the calcium buffering proteins, parvalbumin and calbindin
D28K\textsuperscript{102–104} and they have a high number of the calcium-permeable $\alpha$-amino-3-hydroxy-5-
methyl-4-isoxazolepropionic acid (AMPA) receptors.\textsuperscript{105,106} These two factors make motor
neurons more susceptible to glutamate excitotoxicity than other cells.

Another possible toxic mechanism in ALS is mitochondria dysfunction, which has been
implicated in both fALS and sALS cases. The presence of vacuolated mitochondria and
mitochondrial dysfunction was found in transgenic mice expressing ALS-causing SOD1
mutations.\textsuperscript{107,108} Additionally, mutant SOD1 has demonstrated an increased association with the
mitochondria compared to wildtype SOD1. Therefore, it is possible that the gain of toxic function
of mutant SOD1 is increased mitochondrial association, which leads to mitochondrial dysfunction. Also, the presence of protein aggregates is another common feature found in all cases of ALS and it has been shown that there is inadequate proteosome function in both fALS and sALS cases. Moreover, there are many other toxic features found in ALS models that could be the cause for selective motor neuron death and ALS, for instance impaired axonal transport has been reported in transgenic mouse models and neurofilament aggregation is a common feature found in axonal aggregates of fALS and sALS. Overall, it is uncertain whether any of these observations are the direct mechanistic cause of motor neuron death in ALS or whether some of these features are a result of another primary cause of the disease. It is possible that one toxic mechanism can cause a slew of other toxic properties. Clearly overlap exists between the various hypotheses explaining ALS toxicity and it is likely that not one mechanism is solely responsible for all ALS cases. For instance, the variability seen in ALS patients may be a result of different causes of toxicity. This diversity of damaging features seen in ALS is part of the difficulty in establishing the toxic mechanism as well as treatment for this disease.

**Current Treatment of ALS**

Presently, there are no curative treatments for ALS, therefore therapy is focused on disease management and symptom control with the aim of maintaining the best quality of life possible. This strategy of management employs a multidisciplinary care team made up of neurologists, physical therapists, occupational therapists, speech pathologists, respiratory physicians, nutritionists, psychologists, and social workers, all of which profoundly impact a patient’s quality of life.

Despite a number of clinical trials and advancements in the understanding of ALS, to date, Riluzole is the only approved drug for the symptomatic treatment of ALS. The mechanism
of action of Riluzole is not completely understood, but it has been shown to interfere with N-methyl-D-aspartate (NMDA) receptor response, stabilize the inactivated state of voltage-dependent sodium channels, reduce glutamate release from pre-synaptic terminals, and induce glutamate uptake.\textsuperscript{116} All of these roles help treat glutamate-mediated excitotoxicity and overall results in a modest increase in the lifespan of ALS patients, averaging 3-6 months.\textsuperscript{117–119} Notably, this drug does not cure ALS, hence it may indicate the involvement of other toxic mechanisms besides excitotoxicity within the disease. Overall, a possible explanation for the current failure in therapeutic strategy relating to ALS is the delayed diagnosis of the disease. This leads to treatments being administered in later stages of ALS, which are possibly less effective.

Recent investigations of ALS treatments are focused on the development of gene and stem cell therapies to stop or reverse disease progression. In regards to gene therapy, the use of viral and non-viral vectors to deliver various therapeutic molecules, including growth factors VEGF and IGF1 and neurotropic factor GDNF, to damaged tissues across the blood-brain barrier have demonstrated positive neuroprotective effects in animal models.\textsuperscript{120–123} Also, the use of small interfering RNAs (RNAi) to knockdown genes involved in ALS, like the Fas death receptor, have demonstrated promising results and could potentially be useful in SOD1-ALS cases.\textsuperscript{124} Lastly, a recent study examined the use of an antisense oligonucleotide directed to the hexanucleotide repeat of C9ORF72 in differentiated neurons from a patient carrying the C9ORF72 repeat. The results demonstrated that the oligonucleotide alleviated all toxic phenotypes which include, the presence of RNA foci, dysregulation of gene expression, sequestering RNA binding proteins, and susceptibility to excitotoxicity, without reducing RNA expression levels.\textsuperscript{19}

Currently, stem cell therapies are in the preclinical stage of development and show great promise. For example, one study demonstrated that human neural stem cell grafts alleviated motor neuron disease in SOD1 transgenic rodents.\textsuperscript{125} In another transgenic mouse study,
mesenchymal stem cell transplantation displayed a delay of disease onset and enhanced motor function. Presently, clinical trials injecting ALS patients with fetal-derived neural stem cells are used being used to test the safety and effectiveness of this therapeutic approach.\textsuperscript{125,126} The hope with stem cells is these cells will be able to provide neurotropic factors to degenerating motor neuron cells.

Another promising area of ALS therapeutics lies in the use of small molecules. ALS and other neurodegenerative diseases have a common feature of insoluble protein inclusions, which strongly indicates the occurrence of protein misfolding. One strategy to combat protein misfolding is through the stabilization of the protein’s native structure using small molecule binders. This avenue of treatment is particularly appealing because it targets the underlying mechanism of proteotoxicity rather than symptom treatment. In the case of SOD1, stabilizing the dimer of the protein through the addition of a disulphide bond was shown to reduce aggregation of A4V-SOD1 in vitro.\textsuperscript{127} Hence, the use of stabilizing small molecule binders has a promising outlook in the treatment of ALS and given the alternative strategies of gene and stem cell therapy, small molecule binders may be the easiest method to employ.

\textit{Rationale, Hypothesis, and Specific Aims}

In our laboratory, we believe SOD1’s role as a free radical scavenger and the presence of an electrostatic funnel leading to its active site, put this protein at risk of oxidative damage and mutant SOD1 is more susceptible to this stress and promotes the formation of a native-like, misfolded, aggregate-prone species. We have examined possible pathogenic mechanisms and intermediates and we have demonstrated that oxidation of wildtype and mutant SOD1 can lead to misfolding and aggregation.\textsuperscript{87–89} We have also shown that mutant SOD1 is more misfolded after oxidation than wildtype SOD1 and dimer dissociation is an early step in the SOD1 misfolding process.\textsuperscript{87,89} For these reasons, we believe that stabilizing the SOD1 dimer will lead to the
prevention of monomerization and aggregation of this protein and we believe this approach is a viable therapeutic strategy.

In this thesis, I hypothesize that small molecules that stabilize the dimer of oxidized mutant SOD1 will prevent its misfolding and aggregation. I examine the results of an *in silico* screen identifying small molecules that are predicted to bind to SOD1 native-state. The screen was done on compounds that are either found in traditional medicines or FDA approved, which means they are safe for human consumption. The specific aims of this thesis include the following;

(i) The construction of a SOD1 fusion plasmid that optimizes protein purification.

(ii) Demonstrating that a hit from an *in silico* screen identifying native state binders could prevent oxidation-induced SOD1 misfolding using ANS fluorescence.

(iii) Demonstrating that a hit from an *in silico* screen identifying native state binders could prevent oxidation-induced SOD1 aggregation using right angle light scattering.

(iv) Demonstrate that a hit from an *in silico* screen identifying native state binders could prevent non-oxidative SOD1 misfolding by examining dimer stabilization.

Overall, the discovery of a small molecule that stabilizes the native-state of SOD1 to prevent protein misfolding and aggregation has the potential to be therapeutically valuable to a disease that has seen very little therapeutic progress.

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Chapter Two

Cloning, Expression, Purification, and Characterization of Cu, Zn Superoxide Dismutase and Mutants

SUMMARY

The process of efficient recombinant protein expression and purification is vital to many aspects of biochemistry research. For example, recombinant proteins are important for drug discovery and have the potential to be used as pharmaceutical agents. In this chapter, I describe how I have developed recombinant methodology to produce SOD1, employing a fusion protein consisting of SOD1 fused to a hexa-histidine tag, a venus yellow fluorescent protein, and a TEV protease cleavage site. I used site-directed mutagenesis to create five different ALS-causing mutations in our SOD1 fusion protein. This purification method does not employ the tedious use of multiple column chromatographies nor does it require thermal precipitation procedures, which are employed in traditional SOD1 purification procedures. I analyzed my purified product and found that the recombinant wildtype and mutant SOD1 were highly metallated and maintained enzymatic activity. Overall, I demonstrate that my fusion protein method produces high purity SOD1 in an efficient and cost-effective manner, which makes this construct valuable to the study of SOD1-ALS.

ABBREVIATIONS: SOD1 - Cu, Zn superoxide dismutase, TEV – Tobacco Etch Virus , vYFP – Venus Yellow Fluorescent Protein, PAR - 4-(2-Pyridylazo)resorcinol
INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that selectively targets motor neurons in the brain and spinal cord leading to the progressive paralysis of muscles.\(^1\) In 90% of cases, the cause of the disease is unknown and it is referred to as sporadic ALS (sALS).\(^2\) The remaining 10% of ALS cases involve a genetic component and they are referred to as familial ALS (fALS). Despite the different causes, these cases of ALS are clinically indistinguishable\(^3\) and hence knowledge gained by studying fALS may potentially be applicable to sALS.

One of the major known causes of fALS are mutations in SOD1.\(^4\) Normally, SOD1 functions as an anti-oxidant by catalyzing the dismutase reaction that converts two superoxide anions to one molecule of oxygen and one molecule of hydrogen peroxide. It was demonstrated through the use of recombinant protein that ALS-causing mutations of SOD1 had normal levels of dismutase activity, hence it is believed that mutations of SOD1 lead to ALS through a gain of toxic function rather than a loss of activity.\(^5\) This theory was further corroborated using mouse models that showed the SOD1 knockout mouse did not develop motor dysfunction\(^6\) and mice expressing endogenous SOD1 activity alongside mutant SOD1 still developed ALS.\(^7,8\) The gain of toxic function hypothesis is widely accepted within the ALS field, however the toxic mechanism still remains elusive. While studies involving recombinant wildtype and mutant SOD1 have revealed important structural and mechanistic details regarding this disease, there is still much that needs to be solved. Thus, the purification of recombinant SOD1 continues to be essential to the study of ALS.

Since the initial isolation of haemocuprein (an alternative name for SOD1) from erythrocytes obtained from oxen in the late 1930s,\(^9\) there have been tremendous advances in the area of protein purification. In the last decade, researchers have constructed a number of green
fluorescent protein (GFP) and yellow fluorescent protein (YFP) labeled recombinant wildtype and mutant SOD1 variants. These fusion proteins have been used to explore potential toxic properties of mutant SOD1 using immunofluorescence for localization experiments.\textsuperscript{10–12} More recently, the biophysical properties of these tagged proteins were examined and it was revealed that the GFP tag increased the melting temperature of fusion SOD1 when compared to unlabelled SOD1 protein by 5-6\textdegree C.\textsuperscript{13} This indicates an increased stability of the fusion protein, hence, we have decided to exploit this property in conjunction with other purification developments to create a SOD1 fusion protein optimized for recombinant protein production.

In this paper, we introduce the construction of a new multifunctional tag SOD1 protein. This fusion construct contains the wildtype SOD1 gene inserted into vector DNA containing the coding sequence for a hexa-histidine tag, a venus yellow fluorescent protein (vYFP), and a TEV protease cleavage site. It allows for simple purification through the hexa-histidine tag and TEV cleavage site. Also, it enhances solubility of an aggregate prone protein through the addition of the soluble vYFP, which leads to greater protein yields. Additionally, we created five ALS-causing mutant versions of our fusion protein using site-directed mutagenesis. We demonstrate that the protein purified from our constructs is both properly metallated and active, which strongly indicates that the protein is correctly folded. Overall, this novel fusion protein demonstrates high yield of purified protein of wildtype and mutant SOD1 through its enhanced solubility and it is both easy and cost-effective to purify using a commercially available, reusable Ni-NTA column. These techniques allow for the efficient purification of both wildtype and mutant SOD1 by eliminating the need to purify protein from the insoluble fraction as well as no longer requiring the use of multiple column chromatography techniques.
MATERIAL AND METHODS

Reagents

Commercial wildtype SOD1 was purchased from Sigma-Aldrich (St.Louis, MO, USA). The Ni-NTA resin and PCR purification column were bought from Qiagen (Venlo, Limburg, Netherlands). Protein concentration was measured using the bicinchoninic acid (BCA) assay and the reagents were purchased from Thermo Scientific (Waltham, MA, USA). All other reagents were purchased from Sigma-Aldrich (St.Louis, MO, USA) unless otherwise specified.

Cloning of E.coli expression vector for wildtype SOD1

Our lab has previously constructed a variety of modified DNA vectors that contain venus yellow fluorescent protein (vYFP). We used one of these modified vectors, our pET30a construct that consists of a hexa-histidine tag as well as a vYFP gene, a tev protease cleavage site, and a BamHI restriction site and inserted a wildtype SOD1 gene. The cDNA encoding the human SOD1 protein (a gift from Dr.Janice Robertson) was amplified by PCR using two primers: the forward primer, SOD1 BamHI (+)(5’- CGCGGATCCGACGAAGGC-3’) and the reverse primer, SOD1 XhoI (-)(5’-CCGCTCGAGTTATTGGGCGATCCCAATTACACC-3’). The recognition site for each restriction enzyme is underlined.

The total volume of the PCR reaction was 50µL, which included 100ng of template DNA, 0.5µM of the forward and reverse primer, and 0.5µL of Pfu DNA polymerase (Thermo Scientific, Waltham, MA). The PCR conditions were as follows; an initial denaturation step at 95°C for 3 minutes followed by 25 cycles of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, and primer extension at 72°C for 12 minutes. Lastly, a final extension step was done at 72°C for 10 minutes. The success of PCR was confirmed by electrophoresis on a 1% agarose gel stained with RedSafe (FroggaBio, Toronto, CA) and visualized by UV illumination.
The SOD1 PCR product was purified using a PCR purification column and digested with FastDigest restriction enzymes, BamHI and XhoI at 37°C for 15 minutes (Thermo Scientific, Waltham, MA). This product was again purified using a fresh PCR purification column. Our vector DNA was similarly digested with FastDigest BamHI and XhoI restriction enzymes, followed by a heat inactivation step at 80°C for 10 minutes and subsequently incubated with 1µL of shrimp alkaline phosphatase (Roche, Basel, Switzerland) and purified before ligation. The ligation reaction occurred using T4 DNA ligase in a total volume of 50µL for 24 hours at room temperature (Invitrogen Life Technologies, Carlsbad, CA). The resulting ligation product was transformed into *Escherichia coli* DH5-α cells and the DNA was extracted using a Mini-Prep kit (Qiagen, Venlo, Limburg, Netherlands). The pET30a-(His)$_6$-(vYFP)-(TEV cleavage site)-SOD1 sequence was verified by DNA sequencing from ACGT Corporation (Toronto, ON., Canada).

**SOD1 Mutagenesis**

Five mutations of SOD1 were created using site-directed mutagenesis with our wildtype SOD1 fusion protein. The forward and reverse mutagenic primers are listed in Table 1. The PCR reaction was done in a volume of 50µL and contained 100ng of template DNA, 0.25µM of each primer and 1µL of Pfu polymerase (Thermo Scientific, Waltham, MA). The PCR conditions were as follows; initial denaturation step at 95°C for 2 minutes followed by 16 cycles of denaturation at 95°C for 30 seconds, primer annealing at 50-57°C for 1 minute (temperature depended on the mutation), and primer extention at 68°C for 10 minutes. A 1% agarose gel was stained with RedSafe (FroggaBio, Toronto, CA) to confirm the success of PCR. Sample purification was done using a PCR purification column and the sample was digested at 37°C for 1 hour with Dpn1 (New England Biolabs, Ipswich, MA) before transformation. The mutagenesis was confirmed by complete sequencing of the entire SOD1 gene.
Table 2 – Primers used for constructing various ALS-causing mutants of fusion SOD1

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tbody>
<tr>
<td>A4V-SOD1</td>
<td>5’-TCCGCGACGAAGGTCGTTGCGTTCTGAAG-3’</td>
<td>5’-CTTCAGGACGCACACGACCTTCGTCGCAGA-3’</td>
</tr>
<tr>
<td>I113T-SOD1</td>
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<td>5’-CAGTGTCGGCAGTGATGCATGGTC-3’</td>
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<tr>
<td>G93A-SOD1</td>
<td>5’-GCTGACAAGATGCTGTGGCGATGTG-3’</td>
<td>5’-CACATCGGCACAGCATCTTTGTCAGC-3’</td>
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<tr>
<td>G37R-SOD1</td>
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<td>5’-CAGGACTTCAGTCAGTCATTAAACGACTGAGGGCCTG-3’</td>
</tr>
<tr>
<td>G85R-SOD1</td>
<td>5’-CATGTTGGAGACTTACTGAGCGATGTG-3’</td>
<td>5’-AGCAGTCACATTGCAGTCAGTCAGTCATTAAACGACTGAGGGCCTG-3’</td>
</tr>
</tbody>
</table>

Underlined nucleotides represent the mutated amino acid

Expression and purification of SOD1

The SOD1 fusion protein was expressed in Escherichia coli BL21 cells grown in Luria-Bertani Broth (Novagen, Madison, WI, USA) at 37°C. Protein expression was induced at an OD$_{600nm}$ reading of 0.6 with 0.5mM isopropyl β-D-thiogalactopyranoside (Invitrogen Life Technologies, Carlsbad, CA, USA). At the time of induction 150µM CuCl$_2$ and 150µM ZnCl$_2$ were added to the culture, which was subsequently shaken at 15°C for 24 hours. Bacteria cells were harvested by centrifugation (5,000 g for 15 min at 4°C) and resuspended in 300mM NaCl, 100mM KCl, 50mM Tris-HCl (pH = 7.4), and EDTA-free protease inhibitor (Roche, Basel, Switzerland). The cell suspension was lysed using the EmulsiFlex-C5 (Aveistin) and the sample was centrifuged for 45 minutes at 35,000 rpm using the Beckman L8-70M Ultracentrifuge. The
supernatant was collected and passed through a Ni-NTA column where the His-tag of the protein of interest bound to the nickel on the column. The column was washed three times with 300mM NaCl, 100mM KCl, 50mM Tris-HCl (pH = 7.4), and 40mM imidazole to remove impurities and the fusion protein was eluted from the column with 300mM NaCl, 100mM KCl, 50mM Tris-HCl (pH = 7.4), and 250mM imidazole. In order to remove the imidazole, the elution was dialyzed overnight against 20mM HEPES (pH = 7.4). Next, the fusion protein was cleaved at 30°C for 24 hours using TEV protease that was purified in our lab. The fusion protein:TEV protease ratio was 20:1 and the reaction took place in 20mM HEPES buffer (pH = 7.4). The cleaved sample was passed over a Ni-NTA column and the purified SOD1 was collected in the flow-through, spin-concentrated (EMD Millipore, Billerica MA, USA), and quantified using the bicinchoninic acid assay (BCA).

**PAR Assay**

The metallation status of purified SOD1 was measured using the metal indicator PAR (4-(2-Pyridylazo)resorcinol) as previously described.15 Briefly, 4.25µM SOD1 was denatured using a final concentration of 4.6M guanidine hydrochloride in 20mM HEPES (pH = 7.4). The metal status was detected using 100µM PAR at an absorbance of 500nm using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). When the absorbance stopped increasing, indicating all metals were released from SOD1 and bound to PAR, 0.8mM nitrilotriacetic acid (NTA) was added to the sample and the absorbance was measured. The NTA chelates zinc with a stronger affinity than PAR and therefore in the presence of NTA, PAR will only bind copper. The absorbance of the sample in the presence of NTA was interpolated against a standard curve of 0-14µM CuCl₂ to determine the concentration of copper in solution. The absorbance relating to the copper amount was subtracted from the absorbance of total metal (in the absence of NTA) to determine the absorbance contributed by the zinc released from SOD1. The use of a standard
curve of 0-14µM ZnCl$_2$ allowed the concentration of zinc to be determined by interpolating the absorbance value.

**Remetallation of SOD1**

If the metallation status of either metal was found to be below 80%, the sample underwent a series of dialysis step to metallate the recombinant protein. First, the sample was dialyzed against 2 molar equivalents of CuCl$_2$ in a 40mM sodium acetate buffer (pH=5) for 48 hours at 4°C. This was followed by a second dialysis step against 20mM HEPES (pH=7.4) and 600µM ZnCl$_2$ for 48 hours at 4°C. Lastly, the excess free metals were removed using multiple cycles of dialysis against a 20mM HEPES (pH=7.4) buffer to which a small scoop of Chelex was added.

**SOD1 Activity Assay**

Superoxide dismutase activity of recombinant wildtype and mutant SOD1 was qualitatively examined using activity gels under non-denaturing conditions as previously described.$^{16}$ Briefly, 0.1µg of each protein sample was separated on a 15% native-PAGE. The gel was stained with 275µg/mL nitro blue tetrazolium chloride (NBT), 65µg/mL riboflavin, and 80µL of TEMED in 50mM KPO$_4$ buffer (pH = 7.8) for 45 minutes in the dark. After staining, the gel was exposed to light, which initiates the production of superoxide anion by the stain. The superoxide anion reduces the NBT to formazan blue and stains the gel a dark blue colour. The areas of the gel that have dismutase activity will not reduce NBT and clear spots are related to active SOD1.
RESULTS AND DISCUSSION

*SOD1 Fusion Protein*

The full length coding sequence of wildtype SOD1 was amplified from pcDNA3 with a human SOD1 gene insert. The PCR amplification product was inserted into modified vector DNA containing a hexa-histidine tag (His-tag), a venus yellow fluorescent protein (vYFP), and a TEV protease cleavage site to generate our fusion SOD1 construct, pET30a-(His)$_6$-(vYFP)-(TEV cleavage site)-SOD1. One minor alteration to SOD1 is the addition of glycine and serine to the N-terminal of the protein as a result of the TEV cleavage (Fig.6A). Using NMR, it was confirmed that this addition caused no drastic structural changes to the protein (data not shown). Characterization of the fusion SOD1 using DNA sequencing demonstrated that the target gene was inserted in the correct reading frame of the vector. The resulting fusion protein contained the full length SOD1 with a his-tag, vYFP, and TEV protease cleavage site at its N-terminus site (Fig.6B). The predicted molecular weight of this protein is 48742.29 daltons.

Overall, this SOD1 fusion protein demonstrates many advantages during purification. Firstly, the His-tag allows for separation of the protein from the cell lysate using an inexpensive nickel column that can be regenerated for multiple uses. Additionally, the vYFP aids in protein solubility and prevents poorly metallated SOD1 and mutant SOD1 from entering the insoluble fraction during purification, which increases the protein yield. Also, the vYFP causes the fusion protein to be visible during purification. This allows for a quick visual inspection of the cell pellet for a bright yellow colour to confirm a successful induction and also it allows for easy monitoring of the wash and elution steps from the nickel column. Lastly, the TEV cleavage site permits the SOD1 to be easily separated from the non-SOD1 portion of the fusion protein using the sequence specific cysteine protease, TEV. This is particularly important because both the vYFP and His-tag can interfere with the folding, activity, and biophysical properties of the
protein of interest.\textsuperscript{13,19,20} It is important to remove the large YFP (27kDa) protein from SOD1 (32kDa) in order to ensure that structural interpretation of SOD1 is not influenced by complicating factors. Additionally, the his-tag has high affinity for metal ions and can potentially interfere with the metal binding of SOD1,\textsuperscript{21} which has been shown to lead to structural changes of the protein.\textsuperscript{22,23} In order to avoid these potential problems, it is crucial to be able to separate SOD1 from the remaining portion of the fusion protein and protease cleavage is a commonly used method for tag removal.\textsuperscript{24}
**FIGURE 6:** Fusion construct (His)$_6$-(vYFP)-(TEV cleavage site)-SOD1 (A) Protein sequence of fusion SOD1, which contains a hexa-histidine tag (purple), a venus yellow fluorescent protein (orange), a TEV protease cleavage site (green), and the SOD1 protein (blue). The amino acid residues in black belong to the pET30a vector (B) Schematic diagram representing the recombinant SOD1 fusion protein.
Expression and Purification of recombinant SOD1

The SOD1 fusion gene was expressed in *Escherichia coli* strain BL21 cells and grown in a 2L culture of Luria-Bertani Broth (LB media). During the induction, the culture was supplemented with CuCl$_2$ and ZnCl$_2$. The addition of small amounts of metal was found to be non-toxic to bacterial cells and it improved the metallation state of SOD1, which keeps more protein properly folded and prevents it from entering into the insoluble fraction of the lysate. Hence, this helps increase the protein yield during purification by keeping more protein in the soluble fraction.

Following induction, the bacterial cells were harvested and resuspended in a non-denaturing, high salt buffer supplemented with EDTA-free protease inhibitor. The choice of protease inhibitor is based on keeping SOD1 metallated and therefore properly folded during purification. This will prevent large amounts of protein precipitation once the fusion tag is cleaved. Once cells are lysed and harvested, the supernatant was collected and passed over a nickel column. The His-tag on the fusion protein allowed it to bind to the nickel on the column, while the remaining proteins in the lysate were discarded in the flow-through. A common limitation to His-tag purification is the non-specific binding of metal-binding proteins and proteins rich in cysteine and histidine residues to the nickel column.$^{21}$ For this reason, the column was washed three times with 40mM imidazole in a high salt buffer. Following the wash steps, the fusion protein was eluted off the column with 250mM imidazole and dialyzed overnight.

To examine the effectiveness of the wash steps and the purity of the fusion protein, samples were separated using SDS-PAGE and visualized with Coomassie blue stain (Fig.7A). The wash steps removed some non-specific binding from the column as well as small amounts of fusion protein. Most contaminants eluted from the column during the 250mM imidazole
elution step. To prevent contamination of our protein of interest, the elution was collected in multiple fractions to obtain a clean fraction of the fusion protein. A majority of the fusion SOD1 was collected without the presence of contamination and the total yield was approximately 50-80mg of protein from a 2L culture.

Following dialysis, the fusion protein was cleaved using TEV protease. The cleavage efficiency was low, which we postulate is due to the sandwiching of the TEV cleavage site between two bulky proteins, SOD1 and vYFP. This steric hindrance increases the difficulty of the TEV protease in finding its target site, which leads to lower amounts of product cleavage. We found that increasing the temperature of the TEV cleavage reaction to 30°C optimized the cleavage efficiency without leading to aggregation. Additionally, TEV has the ability to inactivate itself through self-cleavage and over time this lowers the effective concentration of the protease, which contributes to low cleavage efficiency of the fusion protein. In order to overcome this difficulty, fresh TEV is frequently added to the fusion protein sample during its 24-hour incubation.

After TEV cleavage, the product was passed over a Ni-NTA column. This allowed for the separation of cleaved SOD1, which lacks a His-tag, from the other contents of the solution that include uncleaved fusion protein, the non-SOD1 cleavage product, and the TEV protease itself, as all contain a His-tag and will bind to the nickel column. After collecting the flow through and spin concentrating the sample, SDS-PAGE was conducted and the purity of SOD1 was deemed sufficient (Fig.7B). The overall yield of purified SOD1 was determined by BCA assay to be 10-20mg.
FIGURE 7: Purification of recombinant SOD1. (A) The fusion protein was lysed from BL-21 E.coli cells and following high-speed centrifugation, the supernatant was put through a Ni-NTA column. The fusion protein was washed with 40mM imidazole and eluted with 250mM imidazole to obtain a purified fraction of fusion SOD1. (B) The fusion protein was cleaved using TEV protease at 30°C for 24 hours. TEV was added to the fusion protein in intervals to a final ratio of 20 to 1 of protein to TEV. The cleaved product was passed through another Ni-
NTA column and purified SOD1 was collected from the flow-through and subsequently concentrated.

**Mutant SOD1 Fusion Protein**

There are over 170 mutations of SOD1 that can lead to ALS (ALSoD mutation database, as of 1st September 2014) and therefore the ability to produce recombinant mutant SOD1 protein will significantly facilitate ALS research. Using site-directed mutagenesis, we created five fusion constructs of ALS-causing mutations of SOD1. Mutation locations were scattered throughout the protein structure, which included the dimer interface, the β-barrel region, and the metal-binding region (Fig. 8A and 8B). Each mutation confers a unique amount of structural destabilization to SOD1. For instance, mutations in the metal-binding region result in poor copper and zinc binding, lower melting temperature, and more drastic destabilization than mutations found in the dimer interface and β-barrel region.\(^{25}\) Hence, in the study of SOD1-ALS, a disease caused by over a hundred different mutations scattered across all regions of a single protein, having a variety of mutant recombinant protein from the various key areas provides a more thorough examination of the disease.

The expression and purification process of mutant protein was similar to the wildtype protein with one notable alteration. During the TEV cleavage of mutant SOD1, the protein had a higher propensity to aggregate, which was most likely due to lower metallation and increased destabilization of the structure.\(^{22,25}\) To help reduce this aggregation, 100mM NaCl was added to the overnight dialysis of fusion protein following its elution from the nickel column. We were successfully able to purify each mutant using our fusion construct, only the Coomassie stained SDS-PAGE for the purification of A4V-SOD1 is shown (Fig. 8C). Overall, the BCA assay indicated that the protein yield of mutant SOD1 was similar to wildtype SOD1.
FIGURE 8: Purification of recombinant mutant SOD1. (A) Ribbon diagram of SOD1 with various ALS-causing point mutations. Using site-directed mutagenesis a number of different recombinant fusion constructs of mutant SOD1 were created. The various ALS-causing mutation and its location on the protein are listed. (C) Mutant A4V-SOD1 was successfully purified using our fusion protein construct.
The Metallation of Wildtype and Mutant SOD1

SOD1 is a homodimer where each subunit coordinates one zinc ion and one copper ion. Metal loss has been directly indicated in structure destabilization and aggregation of SOD1, therefore it is crucial to have metallated recombinant SOD1 in order to conduct meaningful experiments. Hence, following the expression and purification of wildtype and mutant SOD1, the metallation state of the protein was examined using the metal indicator PAR. Normally, PAR demonstrates a low intrinsic absorbance at 500nm, however, upon complexing to copper or zinc, the absorbance is dramatically increased.

SOD1 samples were incubated with guanidine-HCl, which results in metal release from the protein. The free metals bind to PAR and the absorbance at 500nm was measured. This absorbance value indicates the amount of total metal released by SOD1 and, in order to differentiate between PAR-Cu and PAR-Zn complexes, nitrilotriacetic acid (NTA) was added to the solution following complete metal release. NTA selectively chelates zinc from the PAR-Zn complex, but cannot remove copper from the higher affinity PAR-Cu complex. Therefore, the absorbance at 500nm in the presence of NTA corresponds to the amount of copper in solution and the amount of zinc can be determined by subtracting the amount of copper from the total metal value.

Following purification the metallation status of wildtype SOD1 was found to be approximately 60% metallated with copper and 75% metallated with zinc (Fig.9). Mutant SOD1 was found to be less metallated at around 40-50% for each metal. The metallation of our recombinant protein is low, despite adding free metals to the LB broth during protein expression. We speculate that the presence of a His-tag, which has high affinity for metals, might be preventing the copper and zinc from binding SOD1. Also, NTA is a metal chelator and therefore during column purification with Ni-NTA, it is likely that metals from SOD1 are being stripped
and bound by the NTA, which further decreases the metallation of our purified recombinant SOD1.

In order to insert metals into recombinant SOD1, we devised a procedure that consisted of multiple dialysis steps. First, SOD1 was dialyzed against a buffer of pH 5 in the presence of two molar equivalents of copper. At this low pH, the zinc binding site of SOD1 is unstructured, but the copper site remains intact. This allows copper ions to bind specifically to the copper site of SOD1. Next, the dialysis buffer is changed to a neutral pH with an excess of zinc ions and this allows zinc to bind to its binding site on the protein. After the addition of metals, a series of dialysis steps were done against neutral HEPES buffer and Chelex. This removes the free metals from the sample, which is necessary to prevent metal induced protein aggregation. Following this metallation process, the PAR assay was repeated and both wildtype and mutant SOD1 demonstrated a metallation state of over 80% for each metal (Fig.9).
FIGURE 9: Metal content of purified SOD1. (A) The structure of PAR, a metal chelator that was used to identify the mettallation state of recombinant SOD1. (B) The initial metal status of WT-SOD1 was less than 75% for each metal. A mettallation procedure was conducted using dialysis against CuCl₂ and ZnCl₂ to successfully add metals into the recombinant protein.
Recombinant Wildtype and Mutant SOD1 Possess Dismutase Activity

One method in confirming the proper folding of SOD1 is through the analysis of its dismutase activity. We qualitatively assessed the activity of our recombinant purified protein by monitoring the reduction of nitro blue tetrazolium salt (NBT). Initially, we separated protein samples using native PAGE and then stained the gel with NBT, riboflavin, and TEMED. When the stain is exposed to light it produces superoxide anion, which reduces NBT and stains the gel dark blue. Areas of the gel that have active dismutase will not reduce NBT and the activity is indicated by the presence of clear bands. All purified SOD1 displayed dismutase activity. The level of activity of the recombinant wildtype and mutant SOD1 were comparable to the activity of commercially available wildtype SOD1 (Fig.10). Only poorly metallated SOD1 demonstrated low dismutase activity.
FIGURE 10: The catalytic activity of recombinant SOD1. Samples of 1 μg of each protein were separated using native-PAGE and stained with nitro blue tetrazolium (NBT), riboflavin, and TEMED. In the presence of active SOD1, the NBT substrate will not be reduced to a dark blue colour and clear bands appear. An inverse image of the gel is shown for increased contrast. All recombinant SOD1 constructs demonstrate dismutase activity.
CONCLUSION

In general, the field of protein production and purification has a number of vital research applications, some of which include structural investigations, drug discovery initiatives, and enzyme activity studies. These varied applications require the production of substantial amounts of highly purified protein, hence the need for an effective and economical strategy for protein expression and purification.

In this paper, we introduce a novel multifunctionally tagged SOD1 protein consisting of a hexa-histidine tag, a venus yellow fluorescent protein, and a TEV protease cleavage site. We demonstrate that this protein has high expression in E.coli cells and the purification process is optimized to be simple and economical. Additionally, we used site-directed mutagenesis to create five ALS-causing mutant versions of this fusion protein. We demonstrate that the protein obtained from our constructs is both highly metallated and maintains dismutase activity, which are strong indicators that the purified protein is correctly folded. Overall, these novel fusion proteins allow production of high purity wildtype and mutant SOD1 in a manner that is easy, efficient, and economical and we believe these fusion proteins are a valuable addition to the study of SOD1-ALS.
REFERENCES


Chapter Three
Prevention of SOD1 Misfolding by Small Molecules

SUMMARY

The presence of intracellular protein aggregates in motor neurons is a key feature of ALS. In ALS cases involving mutations of Cu, Zn superoxide dismutase (SOD1), the inclusion bodies contain misfolded intermediates of the SOD1 protein. Mutant SOD1 possesses a high thermal denaturation temperature (96°C) and thus the misfolding of such a stable protein is surprising. Our lab has demonstrated mutant SOD1, and to a lesser degree wildtype SOD1, are susceptible to misfold and aggregate as a result of oxidative stress. Here, we demonstrate that small molecules identified from an in silico screen looking for native-state SOD1 binders, stabilized the native structure of SOD1. We demonstrate that one of these compounds, epigallocatecin-3-gallate (EGCG), stabilized mutant A4V-SOD1 to prevent the protein from misfolding and aggregating in the presence of either oxidative or denaturing stress. These results indicate that EGCG has value that goes beyond its anti-oxidant properties and it may be a promising small molecule with potential therapeutic merit within the field of SOD1-ALS.

ABBREVIATIONS: ALS – Amyotrophic lateral sclerosis, SOD1 - Cu, Zn superoxide dismutase, EGCG - epigallocatecin-3-gallate, PAR - 4-(2-Pyridylazo)resorcinol
INTRODUCTION

Late-onset neurodegenerative diseases are a class of disorders broadly identified by the death of neurons in the central nervous system.\(^1\) Amyotrophic Lateral Sclerosis (ALS) is one such neurodegenerative disease involving the selective loss of motor neurons, causing patients to suffer from symptoms including spasticity, hyperreflexia, progressive muscle weakness, and respiratory failure.\(^2,3\) In approximately 90% of ALS cases, the disease has no known cause and commonly referred to as sporadic ALS (sALS).\(^4\) The remaining 10% of cases can be traced to an identifiable genetic component and these cases are referred to as familial ALS (fALS).\(^4\) In spite of the different causes, ALS cases present with similar pathologies including mitochondrial abnormalities, presence of inclusion bodies, and motor neuron death, making sALS and fALS clinically indistinguishable.\(^5\)

In 1 out of 5 fALS cases, the disease is caused by dominant missense mutations of Cu/Zn superoxide dismutase (SOD1).\(^4\) Human SOD1 is a 32kDa homodimer that functions as an antioxidant by catalyzing the dismutase reaction, converting two molecules of superoxide into oxygen and hydrogen peroxide.\(^6\) Each subunit of SOD1 is composed of 153 amino acids and coordinates a zinc and copper ion.\(^7\) The fully metallated protein is extremely stable with a melting temperature of 85-95\(^\circ\)C\(^8\) and it is found to be enzymatically active in the presence of 8M urea or 4M guanidine-HCl.\(^9\) There are over 100 distinct amino acid mutations along the sequence of the protein that can lead to ALS\(^10\) and a majority of these disease causing mutants possess activity levels similar to wildtype.\(^11\) Hence, it is generally accepted that mutant SOD1 causes ALS through a gain of toxic function rather than a loss of activity. Additional studies using mouse models support this conclusion by demonstrating the knockout SOD1 mouse does not develop ALS\(^12\) and mice expressing mutant human SOD1 still developed motor neuron dysfunction despite the presence of endogenous mouse SOD1.\(^13,14\)
To date, the exact gain of toxic function of mutant SOD1 remains elusive, however, it is clear that protein aggregation is central to the pathology of this disease, as all cases of ALS present with aggregates. Furthermore, it has been shown that the appearance of SOD1 protein aggregation overlaps with the onset of neurodegeneration in ALS transgenic mouse models and mutant SOD1 aggregates more readily than wildtype SOD1 both in vitro and in transgenic mouse models. This gives rise to the hypothesis that the aggregates or aggregate-prone intermediates are the toxic species leading to motor neuron death and ALS. There are many theories regarding how aggregation leads to toxicity including mitochondrial dysfunction, interference with calcium homeostasis, disruption of axonal transport, aberrant binding of apoptotic regulators, glutamate excitotoxicity, and inhibition of the proteasome.

Given the importance of aggregation to the disease, the subsequent question becomes how does mutant SOD1 misfold and form aggregates. It has been shown that various mutations of SOD1 cause a decrease in protein stability, which could ultimately lead to mutants being more susceptible to aggregation. However, many mutant forms of SOD1 are still extremely stable proteins and furthermore some mutations show no difference in terms of stability or metal coordination from wildtype SOD1, yet all mutants are more prone to aggregation. It is possible a denaturant is causing this stable protein to misfold and aggregate. Our lab and others believe that oxidation is the destabilizing mechanism enabling mutant SOD1 to misfold and aggregate. It is possible that SOD1’s role as a free radical scavenger exposes the protein to higher amounts of oxidative stress and due to its long half-life in motor neurons, oxidative damage can accumulate. Our lab has identified covalent modifications to active site amino acids under oxidizing conditions and we have demonstrated these modifications in mutant SOD1 can cause misfolding and aggregation, while wildtype SOD1 remained fairly aggregate-resistant. Furthermore, we found that SOD1 treated with hydrogen peroxide produces
various destabilized SOD1 intermediates, and that mutant SOD1 misfolds more dramatically than *wildtype* SOD1.\(^{37}\) Overall our studies have demonstrated that mutant SOD1 was more susceptible to misfolding and aggregation under an oxidizing stress than *wildtype* SOD1.

Therefore, one possible method in the treatment of SOD1-ALS is to stabilize the structure of the mutant protein to oxidative stress, thus preventing the formation of pathogenic intermediates. In this paper we use an *in silico* drug screen to search for compounds with the potential to bind to native-state SOD1 and possibly stabilize different SOD1 mutations. We demonstrate the A4V-SOD1 mutation, which is considered one of the most severe mutations linked to fALS\(^{7}\), could be stabilized with molecules identified from the *in silico* screen, particularly with the compound epigallocatecin-3-gallate (EGCG). We show that this compound can prevent the misfolding and aggregation of oxidized A4V-SOD1 by stabilizing the structure of the protein rather than through an anti-oxidant role. Overall, from the results of the drug screen we found EGCG to be a compound that demonstrates significant therapeutic potential in treating SOD1-ALS.
MATERIAL AND METHODS

Reagents

All small molecules tested from the drug screen were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) with the exception of Chenodeoxycholic acid, which was purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of each compound were prepared by dissolving each small molecule in 100% ethanol and storing solutions at -20°C. Protein concentration was measured using the bicinchoninic acid (BCA) assay and the reagents were purchased from Thermo Scientific (Waltham, MA, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Cloning and Construction of E.coli expression vector for A4V-SOD1

Our lab has previously cloned venus yellow fluorescent protein (vYFP) into pET23d vector DNA. We have used this same technique to clone vYFP and a TEV cleavage site into other DNA vectors including pET30a. The cDNA encoding the human SOD1 protein (a gift from Dr. Janice Robertson) was amplified by PCR using two primers that included the restriction sites BamHI and XhoI: SOD1 BamHI (+)(5’- CGCGGATCCGCGACGAAGGC-3’) and SOD1 XhoI (-)(5’-CCGCTCGAGTTATTGGGCGATCCCAATTACACC-3’).

The SOD1 PCR product was purified using a PCR purification kit (Qiagen, Venlo, Limburg, Netherlands) and digested with FastDigest BamHI and XhoI. This product was further purified and ligated into our pET30a-(His)_6-(vYFP)-(TEV cleavage site) construct that was similarly digested with BamHI and XhoI restriction enzymes and purified. The resulting pET30a-(His)_6-(vYFP)-(TEV cleavage site)-SOD1 sequence was verified by DNA sequencing from ACGT Corporation (Toronto, ON., Canada). All DNA restriction enzymes were purchased from Thermo Scientific (Waltham, MA, USA).
The A4V-SOD1 mutations were created using mutagenic primers to introduce the desired mutation ((+) 5’-TCCGCGACAAGGCTCGTGCCTGAAG-3’ and (-) 5’-CTTCAGGACGCACACGACCTTCGTCGCGGA-3’). Site-directed mutagenesis was performed on our pET30a-(His)₆-(vYFP)-(TEV cleavage site)-SOD1 construct to introduce the mutation using Pfu polymerase and following the manufacturer’s protocol (Thermo Scientific, Waltham, MA). The mutagenesis was confirmed by complete sequencing of the SOD1 insert.

**Expression and purification of A4V-SOD1 mutant**

Following DNA sequencing, mutant SOD1 was expressed in *Escherichia coli* strain BL21. Bacteria were grown in Luria-Bertani Broth (Novagen, Madison, WI, USA) and protein expression was induced at an OD₆₀₀nm reading of 0.6 with 0.5 mM isopropyl β-D-thiogalactopyranoside (Invitrogen Life Technologies, Carlsbad, CA, USA). At the time of induction, the culture was supplemented with CuCl₂ (150 µM) and ZnCl₂ (150 µM) and shaken overnight at 15°C. Bacteria were harvested by centrifugation (5,000 g for 15 min at 4°C) and resuspended in 300 mM NaCl, 100 mM KCl, 50 mM Tris-HCl (pH 7.4), and EDTA-free protease inhibitor (Roche, Basel, Switzerland). Cells were lysed using the EmulsiFlex-C5 (Aveistin) and the sample was centrifuged for 45 minutes at 35,000 rpm using the Beckman L8-70M Ultracentrifuge. The supernatant was passed through a Ni-NTA column (Qiagen, Venlo, Limburg, Netherlands) and the column was washed three times with 300 mM NaCl, 100 mM KCl, 50 mM Tris-HCl (pH = 7.4), and 40 mM imidazole. The fusion protein ((His)₆-(vYFP)-(TEV cleavage site)-A4VSOD1) was eluted off the column with 300 mM NaCl, 100 mM KCl, 50 mM Tris-HCl (pH 7.4), and 250 mM imidazole and dialyzed overnight against 20 mM HEPES (pH 7.4). The fusion protein sample was cleaved using TEV protease (20:1) at 30°C overnight and the sample was again passed over a Ni-NTA column. The purified A4V-SOD1 is collected
in the flow-through, spin-concentrated, quantified using the bicinchoninic acid assay (BCA) and metallated.

**Remetallation of A4V-SOD1**

The metallation status of purified A4V-SOD1 was measured using the metal indicator PAR as previously described. Briefly, 4.25µM SOD1-A4V was denatured using a final concentration of 4.6M guanidine hydrochloride in 20mM HEPES (pH 7.4) and the metal status was detected using 100µM PAR in the presence and absence of nitrilotriacetic acid (NTA). The NTA chelates zinc with a stronger affinity than PAR and therefore in the presence of NTA, PAR will only bind copper. The absorbance of the PAR-metal complex was measured at a wavelength of 500nm and the metal concentration was interpolated from a standard curve of 0-14µM for each metal.

If the metallation status of either metal was below 80%, the sample was dialyzed against 2 molar equivalents of CuCl₂ in a 40mM sodium acetate buffer (pH 5) for 48 hours at 4°C. A second dialysis step was done against 20mM HEPES (pH 7.4) and 600µM ZnCl₂ for 48 hours at 4°C. The excess free metals were removed by multiple cycles of dialysis in 20mM HEPES (pH 7.4) and a small scoop of Chelex.

**In silico SOD1 binding screen**

The *in silico* SOD1 binding screen was conducted by Anna Cunningham from the Mochly-Rosen laboratory at Stanford University. They screened the SWEETLEAD library of drugs containing both FDA approved and traditional medicine compounds and looked for molecules that bound to native-state SOD1.

**Oxidation of SOD1**

Wildtype and mutant SOD1 were oxidized using 5mM H₂O₂ and 100µM EDTA in 20mM HEPES buffer (pH 7.4). Control samples were prepared by incubating a similar
concentration of protein in 20mM HEPES buffer (pH 7.4). All samples were incubated for 24 hours at 25°C unless otherwise stated. Wildtype SOD1 was purchased from Sigma-Aldrich (St.Louis, MO, USA) and dissolved in 20mM HEPES (pH 7.4), aliquoted, and stored at -20°C.

**Incubation of SOD1 with Small Molecule Binders Identified from In Silico Screen**

To test the effect of small molecules in stabilizing the structure of mutant SOD1, 5-10µM of oxidized and control A4V-SOD1 samples were treated with 0-100µM of SOD1 small molecule binders from the drug screen. The small molecule binders were either added at the beginning of the oxidation treatment or after the 24 hour oxidation treatment and incubated for an additional 3 hours at 25°C.

**8-Anilino-1-napthalene-sulfonic acid (ANS) Binding**

Protein misfolding was detected by adding 100µM ANS to 5µM protein samples in the presence and absence of either SOD1 small molecule stabilizer (0-25µM) or mannitol (0-10mM). The samples were excited at 375nm and emission fluorescence was measured at 490nm using a Photon Technology International QM-1 fluorescence spectrophotometer. All samples were vortexed prior to measurement to resuspend any aggregates. Unless stated otherwise, data was normalized using the following equation:

\[
\frac{[(\text{observed ANS fluorescence}) - (\text{minimum ANS fluorescence})]}{[(\text{maximum ANS fluorescence}) - (\text{minimum ANS fluorescence})]}
\]

Additionally, EC50 values were obtained by fitting data to the following equation;

\[y = A_{\text{min}} + \frac{(A_{\text{max}} - A_{\text{min}})}{(1 + (x/EC_{50})^b)}\]

using Origin 9.

**Right Angle Light Scattering (RALS)**

The presence of aggregates were detected in samples of 10µM A4V-SOD1 that were oxidized for 24 hours at 37°C in the presence or absence of the SOD1 small molecule stabilizer (0-25µM) or mannitol (0-8mM). Scatter measurements were made at an excitation and emission
wavelength of 350nm. All samples were vortexed prior to measurement to dislodge any aggregates. Data was normalized and fitted using the formulas described for ANS dye binding experiments.

**Dimer Dissociation**

The rates of dimer dissociation were measured for samples of 8µM A4V-SOD1 that were either untreated or treated with 100µM of the SOD1 small molecule stabilizer using a cross-linking assay described previously.\(^\text{40}\) Briefly, A4V-SOD1 was unfolded using a final concentration of 2.5M guanidine-HCl (pH 7.5) at 25°C. At various time points, dimeric A4V-SOD1 was cross-linked with glutaraldehyde at a final concentration of 1%. Each cross-linking reaction occurred for 2 minutes at room temperature and then quenched by a 20 times dilution with 200mM Tris (pH 7.5) and 8M urea.

Once all time points were collected, samples were concentrated to a volume of 20µL with microcon centrifugal filter units with a molecular cutoff of 10,000 Da (EMD Millipore, Billerica MA, USA). The total sample was separated using SDS-PAGE and a 12% Tris-glycine gel. Gels were stained with Coomassie Blue R-250 (BioRad, Hercules, CA, USA) and band quantification of the A4V-SOD1 dimer and monomer were done using ImageJ software (National Institutes of Health, Bethesda, MD). Band intensities were plotted as a function of time and fitted to a single-exponential equation using Origin 9.
RESULTS

In silico SOD1 Binding Screen

An in silico screen seeking out native-state binders of SOD1 was conducted by collaborators from the Mochly-Rosen laboratory at Stanford University. They used the Openeye software module FRED to search through a library of over 4000 compounds that were either found in traditional medicines or approved by the FDA, and they discovered over a thousand potential binders of SOD1. They examined 500 molecules that were predicted to bind to the dimer interface of SOD1 (Fig.1A) and they generated all possible three-dimensional conformations of each compound in the library using Openeye Omega. These compounds were docked to two SOD1 dimer interface pockets and they were sorted based on their chemgauss4 score. The chemgauss score is based on shape and chemical complementary groups and it is heavily influenced by potential hydrogen bonding interactions. It is hypothesized that the binding of these compounds could stabilize the structure of mutant SOD1 by strengthening the dimer interaction, which could potentially prevent the protein from misfolding and forming toxic, aggregate-prone intermediates.

Several of these predicted native-state SOD1 binders were purchased based on availability and cost-efficiency (Fig.1B and 11C). Of the compounds purchased, (-)-huperzine A, chenodeoxycholic acid, nicergoline, and benzopurpurine 4B are predicted to bind to SOD1 as shown in the right panel of Figure 11A. The binding site of the three flavonoid compounds, quercetin 3-β-D-glucoside, (-)-epigallocatechin gallate, and quercitrin hydrate, is shown in the left panel of Figure 11A.
(Figure legend on following page)
FIGURE 11: *In silico* SOD1 binding screen. (A) The SOD1 binding screen examined two promising sites of binding in the dimer interface of the protein. The ribbon diagram shows SOD1 in yellow and the binding molecule in blue. Images were produced by Anna Cunningham of the Mochly-Rosen lab at Stanford University using Openeye VIDA (B) Structures of compounds binding to the top pocket of the dimer interface *(left panel of 11A)* (C) Structures of compounds binding to the bottom pocket of the dimer interface *(right panel of 11A)*
In order to test the capabilities of each native-state SOD1 binding compound in reducing SOD1 misfolding, a read-out that differentiates between native-folded and misfolded SOD1 was required. For this reason, we used hydrogen peroxide to induce protein misfolding via a physiologically-relevant oxidative stress and we measured the resulting misfolding using ANS emission fluorescence. Hydrogen peroxide, a normal product of the dismutase reaction, can use the catalytic copper at SOD1’s active site to drive the dismutase reaction backwards and produce free radical species.\textsuperscript{41} These reactive oxygen species cause direct damage to the active site histidine residues by converting histidine to 2-oxo-histidine.\textsuperscript{37} This modification results in reduced metal binding of the protein and considerable conformational changes that can be measured using ANS fluorescence.\textsuperscript{37}

ANS is a charged hydrophobic fluorescent dye that is commonly used to detect partially folded states of proteins through its hydrophobic interactions.\textsuperscript{42} ANS dye binds to hydrophobic regions of a protein. Thus as a protein misfolds, it exposes increased amounts of buried hydrophobic residues at its surface, which results in an increase in ANS binding and a subsequent increase in ANS emission fluorescence.

Structural changes of both wildtype SOD1 and mutant A4V-SOD1 were induced using an overnight incubation of each protein with increasing concentrations of hydrogen peroxide. ANS emission fluorescence was measured as an indication of the amount of misfolding that resulted (Fig.12). Increasing hydrogen peroxide concentrations caused increased misfolding of mutant A4V-SOD1, but demonstrated no detectable misfolding of wildtype SOD1. This is in agreement with mutant A4V-SOD1 being more destabilized than its wildtype counterpart.\textsuperscript{43}
FIGURE 12: Oxidation produced misfolding in A4V-SOD1, but not wildtype SOD1. Samples of 5µM wildtype SOD1 (filled) and 5µM mutant A4V-SOD1 (open) were treated with 0-8mM H₂O₂ in the presence of 100µM EDTA at room temperature for 24 hours. ANS was then added to a final concentration of 100µM, and the emission fluorescence at 490nm was measured. The data is expressed as a ratio of the emission fluorescence of each sample to the emission fluorescence of the untreated sample. ANS fluorescence is visibly increased with an increase in concentration of hydrogen peroxide in the mutant A4V-SOD1 samples, but not in wildtype SOD1 samples. The mutant A4V-SOD1 curve was fitted to the EC50 equation using Origin 9 and the data represents values from a single experimental trial.
Since oxidation of wildtype SOD1 resulted in no observable change in ANS emission fluorescence, we used A4V-SOD1 to assess the effectiveness of the SOD1 native-state binders in reducing oxidation-induced misfolding. Samples of A4V-SOD1 were oxidized overnight with 5mM hydrogen peroxide in the presence of each of the small molecule compounds. Following oxidation, ANS emission fluorescence was measured for each sample and compared to the ANS emission fluorescence of oxidized A4V-SOD1 in the absence of any binding compound (Fig.13). Remarkably, all three of the flavonoid compounds demonstrated a significant reduction of ANS emission fluorescence to levels similar to the unoxidized protein. Additionally, huperzine A and nicergoline demonstrated a statistically significant reduction in ANS emission fluorescence, however, this reduction was not as dramatic as the flavonoid compounds. Overall, this suggests that A4V-SOD1 oxidation-induced misfolding can be inhibited by the presence of certain hits from the in silico binding screen.

In the examination of the three flavonoid compounds, it was discovered that the function of quercetin 3-β-D-glucoside and quercitrin hydrate were unable to be accurately measured with ANS dye due to complications of spectral overlap. An absorbance spectrum of each of the flavonoid compound demonstrates that only (-)-epigallocatechin gallate (EGCG) has no absorbance at the excitation wavelength of ANS (375nm) (Supplementary Fig.1). Therefore, the remainder of this work will focus on the value of EGCG as a SOD1 native-state stabilizing compound.
FIGURE 13: ANS fluorescence of oxidized A4V-SOD1 in the presence of SOD1 native-state binders. Samples of 5µM mutant A4V-SOD1 were oxidized with 5mM H$_2$O$_2$ and 100µM EDTA for 24 hours at room temperature in the presence of 25µM of various SOD1 native-state binders. Following oxidation, ANS was added to a final concentration of 100µM, and the emission fluorescence was measured at 490nm. The data is expressed as a ratio of the emission fluorescence for each small molecule to the emission fluorescence of the unoxidized sample. Values represent means ± SD (n=3); * P < 0.0001.
SUPPLEMENTARY FIGURE 1: Spectral analysis of flavonoid compounds. The absorbance spectrum of 1mM of each flavonoid compound in 20mM HEPES pH 7.5. Only EGCG demonstrated no absorbance at a wavelength of 350nm or higher.
EGCG Does Not Reduce ANS Emission Fluorescence of Oxidatively Damaged A4V-SOD1 by Displacing ANS Molecules

The potency of EGCG was examined through ANS emission fluorescence. Increasing amounts of the small molecule EGCG (0-25µM) were incubated with samples of A4V-SOD1 during a 24-hour oxidation with hydrogen peroxide. The ANS emission fluorescence demonstrated that EGCG reduced the oxidation-induced misfolding of A4V-SOD1 in a dose-dependent manner (Fig.14). Fitting the data of six independent trials, the EC50 value with standard deviation of EGCG with 5µM oxidized A4V-SOD1 was determined to be 2.94±0.50µM. This value suggests that approximately one molecule of EGCG can stabilize one molecule of SOD1 dimer.

In order to demonstrate that EGCG was reducing ANS emission fluorescence in a manner that is indicative of reduced misfolding rather than the displacement of ANS dye, other hydrophobic proteins that bind ANS were examined. ANS emission fluorescence was measured in samples that contained increasing concentrations of EGCG, but replacing A4V-SOD1 with 5µM of BSA or 5µM of molten globule alpha-lactalbumin. Samples of alpha-lactalbumin were dissolved in distilled H2O and 0.1M NaCl at pH 2 and left overnight at 37°C to produce the molten globule state of the protein.44 The molten globule state was verified by comparing ANS emission fluorescence of the protein at pH 2 with the ANS emission fluorescence of the protein at pH 7. It was determined that the fluorescence at the lower pH was 4 times larger than the neutral pH, verifying the molten globule state was formed. When the molten globule alpha-lactalbumin was incubated with EGCG during the 37°C overnight incubation, the ANS fluorescence demonstrated that the small molecule had no effect in reducing protein misfolding (Fig.14).
Additionally, incubation of EGCG with BSA demonstrated a small linear reduction in ANS emission fluorescence (Fig. 14). This reduction is in contrast with the dose-dependent curve seen between EGCG and A4V-SOD1 and it implies that EGCG is likely displacing ANS molecules from BSA, but not from oxidized A4V-SOD1.

Thus this data demonstrates that EGCG did not dramatically reduce the ANS emission fluorescence in molten globule alpha-lactalbumin and BSA, two proteins that have exposed hydrophobic residues and bind to the ANS dye. This suggests that the reduction of ANS emission fluorescence occurring in oxidized A4V-SOD1 in the presence of EGCG is the result of more than a displacement of ANS molecules. Furthermore, it strengthens the case that the small molecule is promoting a conformation of oxidized A4V-SOD1 that exposes less hydrophobic residues at its surface.
FIGURE 14: A comparison of ANS fluorescence of oxidized A4V-SOD1 (filled circles), BSA (open circles), and molten globule alpha-lactalbumin (filled triangles) in the presence of EGCG. Samples of 5µM of each protein were incubated with 0-25µM of EGCG for 24 hours at either room temperature (oxidized A4V-SOD1 and BSA) or 37°C (molten globule alpha-lactalbumin). ANS was added to a final concentration of 100µM, and the emission fluorescence at 490nm was measured. Values for each protein represents the data from a single experimental trial. The small molecule, EGCG, demonstrated no effect in reducing the ANS emission fluorescence for molten globule alpha-lactalbumin, a minimal effect in BSA, and a dramatic reduction in samples of oxidized A4V-SOD1. The curves of BSA and A4V-SOD1 were fitted to the EC50 equation using Origin 9.
EGCG Prevents A4V-SOD1 Misfolding Independent of its Anti-oxidant Function

The small molecule, EGCG, is a potent anti-oxidant found in green tea. Therefore, it is possible that EGCG prevents hydrogen peroxide induced misfolding of A4V-SOD1 through an anti-oxidant role rather than a stabilization of the protein’s structure. To test this possibility, the ANS emission fluorescence was examined in samples where no EGCG was present during the time of oxidation. The A4V-SOD1 samples were oxidized overnight in the absence of EGCG and three hours prior to measuring ANS emission fluorescence, varying concentrations of EGCG were added to these samples. The occurrence of oxidative damage was confirmed by comparing the ANS emission fluorescence of a sample of unoxidized A4V-SOD1 with the fluorescence of oxidized A4V-SOD1. The oxidized sample demonstrated a fluorescence value that was 2-fold larger than the unoxidized sample, confirming the presence of oxidatively-damaged, misfolded A4V-SOD1. It was discovered that adding EGCG to A4V-SOD1 post-oxidation still resulted in a decrease in ANS emission fluorescence, albeit to a slightly lesser extent than adding EGCG during the time of oxidation (Fig.15). These results support the notion that EGCG prevents oxidation-induced A4V-SOD1 misfolding through a mechanism that is mostly independent of its anti-oxidant activity. Additionally, it demonstrates that following oxidative damage and protein misfolding, EGCG was able to promote some structural refolding and stabilization of A4V-SOD1.

Further confirmation that EGCG prevents oxidative misfolding of A4V-SOD1 independent of its anti-oxidant activity was explored using the free-radical scavenger mannitol. Both mannitol and EGCG act as anti-oxidants through their ability to scavenge free radicals. Therefore, if EGCG was preventing oxidative misfolding of A4V-SOD1 through its anti-oxidant function, mannitol should demonstrate a similar protection. The ANS emission fluorescence of A4V-SOD1 oxidized in the presence of 0-10mM mannitol demonstrates that
despite the oxidative protection provided by mannitol, there was no reduction of A4V-SOD1 misfolding (Fig.16A). This is most likely due to the fact that the free radicals produced by SOD1 occur at the active site, therefore damage to the active site histidines is caused by close proximity of free radicals and the effect of added anti-oxidants that are distal to the active site would be minimal. This further supports the notion that EGCG reduces A4V-SOD1 misfolding through a mechanism that is independent of its anti-oxidant activity.

*EGCG Prevents Aggregation of Hydrogen Peroxide Damaged A4V-SOD1*

One consequence of protein misfolding is the formation of aggregates. In order to evaluate the effect of EGCG on the aggregation of A4V-SOD1 caused by oxidative damage, right-angle light scattering was measured. First, samples of 10µM A4V-SOD1 were oxidized overnight with 5mM hydrogen peroxide at 37°C to form aggregates. The presence of aggregates was verified by comparing the right-angle light scattering of oxidized A4V-SOD1 to unoxidized A4V-SOD1. The amount of scattered light is proportional to both the size and number of aggregates present. Thus, the increase in scattering of the oxidized sample implies the formation of either an increased number of aggregates or the presence of larger aggregates. To test the effect of EGCG on A4V-SOD1 aggregation, varying concentrations of 0-25µM EGCG were added to A4V samples during the overnight oxidation. The right-angle light scattering of these samples demonstrated a dose-dependent decrease in signal with increased amounts of EGCG (Fig.16B). The data of six independent experiments were fit to the EC50 equation and the mean EC50 value with standard deviation of EGCG and 10µM A4V-SOD1 was found to be 6.22±0.69µM. This value is comparable to the EC50 value determined by ANS emission fluorescence and these results demonstrate that EGCG can prevent oxidation-induced aggregation of A4V-SOD1.
Additionally, right-angle light scattering was measured for A4V-SOD1 samples that were oxidized in the presence of 0-10mM mannitol. In agreement with the ANS emission fluorescence data, mannitol had no significant effect in reducing the right-angle light scattering of oxidized A4V-SOD1 (Fig.16B). In summary, these results demonstrate that the presence of EGCG is able to reduce both misfolding and aggregation of oxidatively damaged A4V-SOD1 in a manner that is independent of its anti-oxidant function.
FIGURE 15: Effect of EGCG on ANS fluorescence of H$_2$O$_2$-treated mutant A4V-SOD1. Samples of 5µM A4V-SOD1 were either oxidized in the presence (filled) or absence (empty) of 0-25µM EGCG. Oxidation occurred for 24 hours at room temperature with 5mM H$_2$O$_2$ and 100µM EDTA. Samples that were oxidized in the absence of EGCG were then incubated with 0-25µM EGCG for 3 hours at room temperature prior to the addition of ANS to a final concentration of 100µM. Emission fluorescence was measured at 490nm and both curves represent data from a single trial. EGCG produced a dose-dependent reduction in ANS emission fluorescence in both sets of data and each curve was fitted to the EC50 equation using Origin 9.
FIGURE 16: Mannitol demonstrates no effect in reducing misfolding or aggregation of oxidized A4V-SOD1. (A) Samples of 5µM A4V-SOD1 were oxidized with 5mM H₂O₂ and 100µM EDTA at room temperature for 24 hours in the presence of either 0-25µM EGCG (filled) or 0-10mM mannitol (empty). ANS was added to a final concentration of 100µM and emission fluorescence was measured at 490nm. (B) Samples of 10µM A4V-SOD1 were oxidized with 5mM H₂O₂ and 100µM EDTA at 37°C for 24 hours in the presence of either 0-25µM EGCG (filled) or 0-8mM mannitol (empty). Right angle light scattering was measured at 350nm. Both sets of data represent a single experimental trial and the data from these experiments was plotted on double x-plots and fitted to EC50 curves in Origin 9.
The A4V-SOD1 Dimer Interface was Stabilized by EGCG

To test whether the mechanism of EGCG reduces oxidation-induced misfolding and aggregation of A4V-SOD1 through dimer stabilization, the dissociation of the dimer of A4V-SOD1 was examined in the presence of guanidine-HCl. An 8µM sample of A4V-SOD1 in the absence and presence of 100µM EGCG was incubated with 2.5M guanidine-HCl at 25°C. A 40µL sample of the mixture was removed at varying time points and the dimer in the aliquot was cross-linked with 1% glutaraldehyde for 2 minutes before the reaction was stopped with 200mM Tris and 8M urea. The samples from the varying time points were run on an SDS-PAGE gel and stained. The band intensity of dimer to total protein of each time point was measured using Image J and this data was fitted to a single-exponential curve. The results demonstrate that in the presence of EGCG the dissociation of the A4V-SOD1 dimer was visibly slower (Fig.17). These results suggest that EGCG can stabilize the dimer of A4V-SOD1 in the presence of guanidine-HCl. In this experiment, the use of a denaturant that did not damage the protein through an oxidative mechanism was intentional. It helps to further validate that the mechanism in which SOD1 prevents the misfolding and aggregation of A4V-SOD1 occurs through a stabilization of the protein structure rather than through an anti-oxidant mechanism.
FIGURE 17: The rate of dimer dissociation of A4V-SOD1 was determined to be slower in the presence of EGCG. Dimer dissociation was measured by covalently cross-linking 8µM of A4V-SOD1 during various stages of its unfolding with 2.5M guanidine-HCl. Samples were treated one hour prior to unfolding with 100µM EGCG (empty circles) or left untreated (filled circles). Dimer band intensity was quantified on a Coomassie-stained SDS-PAGE gel, normalized, and fitted to a single-exponential equation in Origin 9. Data represents a single experimental trial.
DISCUSSION

As life expectancy increases, age-related illnesses will become more prevalent and incidences of late-onset neurodegenerative diseases are expected to rise. Today, treatments for these classes of diseases are limited to temporary relief of symptoms rather than intervention that affects the disease course.\textsuperscript{47} The aim of this \textit{in silico} binding screen was to identify small molecules that have the potential to bind and stabilize the native structure of SOD1, to prevent the protein from misfolding and forming toxic, aggregate-prone intermediates. Here, we used ANS fluorescence to examine the capabilities of seven compounds that were predicted to bind to the dimer interface of native-state SOD1. We found that the small molecule, EGCG was effective in reducing the oxidation-induced misfolding of mutant A4V-SOD1. Similarly, the results from right-angle light scattering experiments demonstrated EGCG could decrease the aggregation of oxidatively stressed A4V-SOD1. The mechanism of action of EGCG was shown to be independent of its anti-oxidant function by demonstrating that another anti-oxidant, mannitol had no effect in preventing the misfolding and aggregation of oxidized A4V-SOD1. Lastly, by examining the dissociation of the A4V-SOD1 dimer using the non-oxidative denaturant, guanidine-HCl, we demonstrated that the rate of A4V-SOD1 dimer dissociation was slower in the presence of EGCG. Together, these results indicate that EGCG is able to stabilize the structure of mutant A4V-SOD1 allowing for the stronger association of the dimer and consequently resulting in decreased amounts of protein misfolding and aggregation.

\textit{The Anti-aggregation Capabilities of EGCG}

The compound EGCG is a naturally occurring polyphenol found in green tea. In addition to its anti-oxidant role, this small molecule has demonstrated tremendous potential as an anti-aggregation agent. EGCG can prevent the formation of amyloid fibrils of α-synuclein and amyloid-β (Aβ) by promoting the formation of non-toxic stable oligomers.\textsuperscript{48–51} Efforts have
been made regarding the elucidation of the molecular mechanism by which EGCG prevents the formation of Aβ fibrils. Using solution-state NMR experiments, it has been demonstrated that EGCG interacts with the aromatic hydrophobic core of the Aβ peptide to form stable oligomers that are not able to adopt the β-sheet conformation of amyloid fibrils.\textsuperscript{51,52} Thus, it is proposed that EGCG prevents Aβ nucleation and fibril growth through disruptive steric interactions.\textsuperscript{51,52} This suggests that the hydrophobic interactions of EGCG is a possible common mechanism for the anti-aggregation property this compound demonstrates in a wide variety of proteins including; α-synuclein,\textsuperscript{48,50} transthyretin,\textsuperscript{53,54} huntingtin,\textsuperscript{55} and now mutant SOD1.

In agreement with our findings that EGCG stabilized the dimer interface of SOD1, the tetramer of transthyretin was also shown to be stabilized in the presence of this small molecule.\textsuperscript{53} Using x-ray crystallography and a transthyretin stabilization assay, it was shown that EGCG had three distinct binding sites to the protein and its binding resulted in tetramer stabilization, which caused a decrease in appearance of aggregate prone transthyretin monomers and oligomers.\textsuperscript{53} Additionally, the aggregate susceptible protein found in Huntington’s disease, huntingtin, demonstrated that its aggregation could be prevented using EGCG or (−)-gallocatechin 3-gallate (GCG), but not using two other related compounds, (−)-gallocatechin (GC) and (−)-epigallocatechin (EGC), which lack a gallate moiety.\textsuperscript{55} Similarly, the gallate moiety was found to be important in the anti-aggregation role of EGCG in α-synuclein and Aβ aggregates.\textsuperscript{50} These studies suggest that EGCG may use its gallate moiety to form hydrophobic interactions with proteins and consequently lead to the stabilization of various non-toxic intermediates of aggregate prone proteins. Our lab is currently investigating the binding properties of EGCG to SOD1 using NMR techniques in order to validate the dimer stabilization properties of this compound.
Pharmacological Properties of EGCG

Overall, green tea derivatives like EGCG are attractive drug candidates because they are naturally occurring compounds that are safe for human consumption and can cross the blood-brain barrier in mammals. A preliminary study involving TTR amyloidosis patients demonstrated that taking a daily dose of 500-700mg of EGCG (approximately 1.5-2L of green tea) for one year resulted in a decrease in cardiac wall thickness in 86% of treated patients. Additionally, studies involving mice models of Alzheimer’s disease showed a decreased amount of plaque load and an increase in cognitive function from the oral administration of 50mg/kg body weight of EGCG. It has been shown that a dose of 75mg/kg body weight results in a plasma concentrations close to 1µM EGCG in mice. Relating this to humans, a similar plasma level is achieved through the consumption of approximately 450mg of EGCG and a dose of up to 800mg daily was shown to be safe for human consumption. Hence, the use of EGCG in the treatment of neurodegenerative diseases appears promising. Nevertheless, the positive effect seen in response to EGCG treatment may not be entirely due to the anti-aggregation properties of the compound. EGCG has many beneficial activities including radical scavenging, metal chelation, and reduction of oxidative stress, which also may contribute to decreased levels of plaques.

Despite these promising effects, one widely recognized problem with EGCG treatments is the variability of this compound’s bioavailability. Some issues include rapid metabolism within the body and conjugation by the liver (ie.sulfation, glucuronidation, and methylation). It is possible to optimize the bioavailability of this compound through a diligent oral routine. For instance, taking the compound after an overnight fast increases its absorption at the gut and the simultaneous intake of vitamin C, piperine, and fish oil have all shown increased levels of
EGCG absorption. Additionally, the encapsulation of EGCG in nanoparticles has shown increased levels of uptake and longevity of this compound within the body.

The Role of EGCG within SOD1-ALS

The potential use of EGCG as a stabilizing molecule for mutant SOD1 within the field of ALS could be groundbreaking by providing a treatment that affects the disease course. Investigation of the therapeutic value of this compound in ALS models is just beginning. One early study showed that the intraoral treatment of transgenic mice containing the G93A-SOD1 mutation with 10mg/kg body weight of EGCG both delayed disease onset and show prolonged lifespan. Thus, this compound demonstrates potential therapeutic value within the SOD1-ALS field.

Overall, the results of the in silico binding screen proved to be valuable in predicting potential SOD1 native-state binders. It should be noted that despite the little effect seen in the non-flavonoid compounds, (-)-huperzine A, chenodeoxycholic acid, nicergoline, and benzopurpurine 4B, these hits may still prove to be effective in stabilizing other mutations of SOD1. It is clear that not all SOD1 mutations behave similarly and therefore a multifaceted therapy may be necessary for the treatment of SOD1-ALS. In general, the findings of this study indicate for the first time that EGCG acts to stabilize mutant SOD1 to prevent its misfolding and aggregation. These results support the existing body of evidence that demonstrate the anti-aggregation properties of EGCG.
REFERENCES


Chapter 4

Discussion

Aggregation and Protein Misfolding in Neurodegenerative Diseases

In the development of long-term treatments of neurodegenerative diseases (NDDs), discerning the causative pathogenic mechanism is crucial for developing therapies that go beyond the management of the downstream effects of neuronal degeneration. One of the hallmark features of NDDs, which include Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and amyotrophic lateral sclerosis, is the accumulation of protein-based deposits. In each of these diseases a different protein is associated with aggregate formation, but a common link appears to be protein destabilization. In the instance of the non-neuronal condition amyloidosis, the transthyretin protein must be partially destabilized and expose aggregate prone sequences that can self-interact in order to produce protein inclusions.\(^1\) Additionally, many other amyloid diseases are associated with mutations or irregular cleavage of a protein that results in a destabilized native structure that exposes aggregate-prone sequences to its surface.\(^2,3\)

In the specific case of ALS, there are a number of different causes related to the disease. Mutations in proteins of all sorts of functions have been linked to ALS\(^4\) as well as a majority of ALS cases have an unknown sporadic onset.\(^5\) Despite the different causative effects, the pathology remains the same, as all ALS cases present with protein deposits in motor neurons, impaired mitochondria, and motor neuron degeneration.\(^6\) In regards to the various protein mutations that lead to ALS, SOD1 is the oldest and most studied known cause of the disease.\(^7\) In SOD1-ALS cases, it has been demonstrated that the onset of neurodengeneration in transgenic mouse models corresponds to the appearance of protein aggregation.\(^8\) Additionally, mutant SOD1 has been shown to be more aggregate-prone than wildtype SOD1 both \textit{in vitro} and in transgenic mice models.\(^9\)
leads to the hypothesis that aggregates are the toxic species in ALS and other NDDs, however this view has come under some debate. Findings suggest that the burden of aggregates does not correlate with neuronal decline\textsuperscript{10,11} and in one study where Aβ plaques were removed through immunization, patients showed no reversal or arresting of cognitive decline.\textsuperscript{12} These results are rationalized through the suggestion that it is not aggregates that are the toxic species in NDDs, but instead a more soluble, aggregate-prone intermediate.\textsuperscript{13,14}

Focusing on SOD1-ALS, our lab has demonstrated that by use of denaturants, mutant SOD1 can form soluble, aggregate-prone intermediates.\textsuperscript{14–18} These intermediates demonstrate loss of zinc and copper binding, dimer dissociation, and increased exposure of hydrophobic residues to the surface of the protein.\textsuperscript{14,16} Furthermore, when wildtype SOD1 was treated with similar denaturants, it demonstrated a lesser degree of structural destabilization and did not populate the same intermediates as seen with mutant SOD1.\textsuperscript{14–18} Therefore, one potential method in the treatment of SOD1-ALS is a strategy that stabilizes the native structure of the protein to prevent it from misfolding and forming toxic aggregate-prone species.

\textit{Treatment of SOD1-ALS using Native-state Stabilizers}

In the field of ALS, treatment of the disease is an area that has seen little progress. One contributing factor is the SOD1-ALS mouse is an overexpression model. This mouse model does demonstrate progressive neurodegeneration akin to the human disease, however, not all pathological features are the same. For instance, the presence of vacuolated mitochondria is an early feature in the transgenic mouse that is not seen in human patients. This misleading pathogenic feature can cause successful treatments in mice to be unsuccessful in human patients. The use of a knock-in mouse model may add more value to the ALS field.

Another complicating factor in ALS treatment is the time of drug administration. Most patients will begin therapeutic treatments at the time of disease onset. At this point aggregates
are present and motor neuron degeneration has already occurred. Treatments that have shown
benefits when administered pre-symptomatically will likely be ineffective once pathogenic
mechanisms are triggered, at best they may slow progression or weaken severity. In a disease
that has seen very little therapeutic benefit, a mild improvement in lifestyle would bring some
optimism.

In contrast to Alzheimer’s disease and Parkinson’s disease, where natively unfolded
protein leads to aggregate formation,\textsuperscript{19} SOD1-ALS is caused by a stable protein. Therefore
therapeutic strategies of these disorders should differ. In the case of Alzheimer’s and
Parkinson’s disease, an approach that reduces the aggregate intermediate would be more
effective than attempting to stabilize an already unfolded protein. In regards to ALS, where a
denaturing stress is required to trigger misfolding and aggregation,\textsuperscript{14,17,18} a treatment that
attempts to stabilize the native structure of the protein may prove effective.

I have demonstrated that one particular flavonoid compound, epigallocatechin-3-gallate
(EGCG) possesses stabilizing properties towards mutant SOD1 treated by a denaturing stress.
More specifically, I have shown that EGCG can inhibit SOD1 misfolding and aggregation
induced by oxidative stress and slow dimer dissociation caused by a chemical denaturant.
Surprisingly, EGCG has previously been tested in ALS models. In 2004, Koh et al.
demonstrated using a motor neuron-neuroblastoma cell line expressing the ALS-causing
mutation G93A-SOD1 that EGCG could protect cells against oxidative stress by acting
upstream of apoptotic signaling pathways.\textsuperscript{20} This study was done with the intention of
examining the anti-oxidant properties of EGCG and the mechanism by which it acts. They
speculate that given the important role of oxidation in neurodegeneration that this compound
would be beneficial via its anti-oxidant properties. In 2006, two independent studies using the
G93A-SOD1 transgenic mouse models showed that EGCG both delayed disease onset and
prolonged survival time by a couple of weeks.\textsuperscript{21,22} It was again demonstrated that apoptotic signaling pathways were downregulated in mice treated with EGCG.

It is very possible that EGCG can benefit ALS patients by reducing oxidative stress. The use of other antioxidants, like coenzyme Q10 have shown mild benefits in ALS mice models.\textsuperscript{23} On the other hand, vitamin E (\(\alpha\)-tocopherol), an extremely potent antioxidant had no therapeutic effect in ALS patients.\textsuperscript{23} Although, this variation may be due to issues crossing the blood brain barrier, the possibility that the anti-apoptotic properties of EGCG may be a downstream effect of mutant SOD1 structure stabilization has potential. In support of the structural stabilization mechanism of EGCG, I have demonstrated that the effect of this compound on reducing misfolding and aggregation of mutant SOD1 is independent of its anti-oxidant role. I examined the effect of EGCG on dimer dissociation that was induced using a chemical denaturant and discovered that the small molecule could slow down dimer dissociation. Additionally, I showed that EGCG could reduce misfolding in mutant SOD1 that was oxidatively-damaged prior to drug treatment and the effect of another antioxidant, mannitol, did not show reduction of either aggregation or misfolding of oxidized mutant SOD1.

Therefore, a possible mechanism of action of EGCG is that this small molecule can stabilize the native structure of SOD1 and prevent the downstream effects of its misfolding, which include the activation of apoptotic pathways. Another possibility is that EGCG can act to both stabilize the structure of oxidatively-damaged SOD1 to prevent its misfolding and aggregation and function as an anti-oxidant to reduce oxidative stress levels in cells. In both cases, EGCG has beneficial effects in reducing SOD1 misfolding, either directly through binding and stabilization of the native structure of the protein or indirectly by reducing levels of oxidative stress in the cell and at the protein binding site through its scavenging capabilities.
Another particularly interesting aspect of this compound, EGCG, is that it has been shown to prevent aggregation of other proteins involved in neurodegeneration. EGCG can bind directly to native unfolded proteins and prevent the formation of toxic aggregate intermediates of Aβ in Alzheimer’s disease\textsuperscript{24,25}, α-synuclein in Parkinson’s disease\textsuperscript{26,27}, and huntingtin in Huntington’s disease\textsuperscript{28} by forming non-toxic oligomers. Additionally, using \textit{in vitro} and cell culture systems, treatment of the protein responsible for amyloidosis, transthyretin (TTR) with EGCG demonstrated improved stability of the TTR tetramer leading to alteration of the toxic intermediate species and reduction in aggregation\textsuperscript{29,30}. Furthermore, in transgenic mice expressing mutant TTR, a clearance of aggregates in response to EGCG treatment was demonstrated\textsuperscript{31}. This study led to preliminary examination of EGCG in TTR amyloidosis patients. Here it was discovered that after one year of treatment with a daily intake of 500-700mg of EGCG, 86% of patients demonstrated a decrease in cardiac wall thickness and mass, which is indicative of reduced TTR deposits and increased clearance of TTR amyloid\textsuperscript{32}. While the anti-oxidant properties of this compound may be responsible for some of the benefits discussed, \textit{in vitro} experiments involving the huntingtin protein demonstrated that the gallate moiety of EGCG was a necessary component of its inhibitory effect on the formation of toxic aggregate intermediates\textsuperscript{28}. This indicates that the hydrophobic binding of EGCG to misfolded proteins is what drives its anti-aggregation role and not its function as an anti-oxidant.

\textbf{Nutraceuticals in Neurodegenerative Diseases}

The compound EGCG is the major occurring polyphenol found in green tea\textsuperscript{31}. Its use in therapeutics is enticing because it is a natural compound that is well-tolerated for human consumption\textsuperscript{28}. Hence, in the treatment of chronic diseases like ALS, where medication would be ingested on a daily basis over a long period of time, natural compounds are well suited for this task. The application of natural compounds, like polyphenols, in the treatment of diseases
falls under the definition of a nutraceutical. In 1989, Dr. Stephen DeFelice coined the term nutraceutical from the words “nutrition” and “pharmaceutical” to describe a food or part of a food that contains health benefits, including the treatment or prevention of a disease. In general, polyphenols are a group of aromatic compounds containing one or more phenolic hydroxyl groups. One theory attempting to explain the anti-aggregation properties of polyphenols suggests the aromatic rings of these compounds compete with aromatic residues on aggregate prone proteins and cause steric hindrance in the formation of toxic oligomers. Another hypothesis previously described, which is more likely to explain EGCG’s contribution to increased stability and anti-aggregation properties in SOD1 models, is that the phenolic hydroxyls of polyphenols may interact with hydrophobic amino acid residues of proteins. This could prevent toxic oligomers from forming as well as stabilize the native-structure of proteins. In the case of SOD1-ALS, destabilization of SOD1 native structure can lead to metal loss in the protein and increased metal concentrations have been linked to aggregation. Hence, a possible outcome of stabilizing the structure of SOD1 is reduced aggregation. Lastly, the antioxidant properties of polyphenols and the metal-scavenging properties of EGCG could also contribute to the effectiveness of these compounds in treating neurodegeneration.

To date, there are a number of polyphenol compounds that have been associated with improvements in neurodegenerative diseases. Curcumin, which is a major component of turmeric, has been linked to improved cognitive function during old age. It is only in the past decade that mechanistic studies involving curcumin were investigated. Studies revealed that curcumin could inhibit aggregation of a number of proteins leading to their decreased cytotoxicity and solid-state NMR revealed that the methoxy or hydroxyl group of curcumin...
can interact with specific residues of the Aβ fibril. Another polyphenol, resveratrol, which is found in red wine has been reported to have protective effects in neurodegenerative models. Resveratrol can inhibit Aβ fibril formation as well as remodel soluble oligomers, fibrillar intermediates, and amyloid fibrils into non-toxic species. Additionally, polyphenols from coffee, olive oil, and traditional Chinese herbs have all demonstrated anti-aggregation capabilities as well as other non-polyphenol nutraceuticals including small molecules like alkaloids, glycosides, and phenazines. The non-polyphenol compounds contain one or more aromatic rings, which are most likely key to their anti-aggregation function. Overall, mechanistic studies are just beginning to shed insight into the therapeutic potential of these compounds, however as preliminary studies are conducted there seems to be a promising future for nutraceuticals in neurodegenerative treatments.

**Future Directions**

In the study of SOD1-ALS, therapeutics is an important area of investigation that has been lagging. Mutations in SOD1 have been linked to ALS for two decades, however only one drug, Riluzole, has been approved in the treatment of this disease. I have demonstrated for the first time that the polyphenol, EGCG, has SOD1 stabilizing abilities that lead to the prevention of misfolding and aggregation of this protein in response to denaturing stress. In the development of EGCG as a therapeutic compound in SOD1-ALS, the bioavailability of this small molecule becomes crucial. Using a number of animal models it has been confirmed that EGCG does cross the blood-brain barrier, specifically the rat model showed that after consuming 500mg/kg body weight of EGCG, the concentration of the small molecule in rat brain tissue samples was determined to be 0.5nmol/g of rat brain and the plasma concentration was determined to be 12.3nmol/mL. In another study, healthy human volunteers who ingested 800mg of EGCG were found to have a plasma concentration of EGCG of
3.40±2.97µmol/mL.\textsuperscript{54} It has been estimated that the concentration of SOD1 in motor neurons is approximately 40µM\textsuperscript{17} and based on our \textit{in vitro} studies to determine the EC50 value for 5µM A4V-SOD1, the concentration of EGCG required to pass the blood brain barrier to be effective as a treatment should be much higher. Therefore, investigations of improved bioavailability of EGCG could offer potential therapeutic benefits for ALS patients.

Currently, an easy to implement method to improve the bioavailability of EGCG is adhering to an optimized oral routine. For instance, ingesting EGCG with vitamin C, piperine, and fish oil have all shown increased levels of EGCG absorption.\textsuperscript{55}

Another method to enhance the bioavailability of EGCG is through structure-activity relationship studies, which is the examination of the relationship between the structure of a molecule and its biological activity. Structure-activity relationship focused studies are used to determine which chemical groups are responsible for a certain biological effects. The use of this information can then be applied in creating a synthetic compound with improved potency and bioavailability. Studies on EGCG have revealed that when the gallate moiety or the 3-hydroxyl group of the trihydroxyphenyl ring are removed, the potency of EGCG is reduced, but not lost.\textsuperscript{56} Only if both groups are removed is the activity of EGCG completely abolished.\textsuperscript{56} These results help identify what structural component of EGCG is the essential factor for its activity and given this knowledge, either a compound in nature or a synthetic compound can be made to optimize both potency and bioavailability. Also, a second \textit{in silico} screen can be done to identify compounds with the presence of therapeutic functional groups.

In addition to improvements in its bioavailability, many molecular studies should be done to gain a better understanding of the anti-aggregation properties of this small molecule. Firstly, the binding of EGCG to SOD1 needs to be validated. This is currently being examined in our lab through the use of NMR and the initial results are promising. Preliminary results show
that wildtype SOD1 appears to undergo spectral changes in the metal binding region and the dimer interface in the presence of EGCG. Once investigations are completed on wildtype SOD1, we will look at the effect of EGCG on various SOD1 mutations. These studies will not only confirm EGCG binding to SOD1, but they could potentially provide further insight into the mechanism of how EGCG prevents misfolding and aggregation of mutant SOD1.

Another key component in the examination of EGCG as a therapeutic treatment in SOD1-ALS is the ability of the molecule to reach its target. If it is determined that the binding of EGCG to SOD1 occurs through hydrophobic interactions between the compound’s gallate moiety and exposed hydrophobic amino acid residues of the protein, it is likely that many binding sites exist in a cellular environment that would compete with EGCG binding to SOD1. The binding affinity between EGCG and SOD1 can be measured using isothermal titration calorimetry, which relates binding affinity to the change of heat that occurs during binding. A strong binding affinity indicates a more specific interaction between the two components and an increased chance that EGCG can find its target protein in a cellular environment. The binding affinity between EGCG and SOD1 can be compared to the binding affinity of EGCG and commonly found hydrophobic proteins like casein. Ideally, EGCG will demonstrate specificity towards SOD1, however, if this is not the case, cellular studies and animal models may still show a positive response towards the small molecule despite non-specific binding.

For instance, EGCG is a naturally occurring compound that has been shown to be safe for human consumption at a daily dose of 800mg.\textsuperscript{32,54} This amount may be enough to bind to non-specific targets as well as to SOD1. Also, the effect of EGCG in the clearance of TTR aggregates in humans shows a therapeutic response towards the small molecule and this implies that EGCG faced minimal interference from non-specific binding to hydrophobic proteins.\textsuperscript{32} Additionally, limited studies of EGCG in mouse models of G93A-SOD1 have demonstrated a
therapeutic response towards the small molecule.\textsuperscript{21,22} This may indicate EGCG was able to bind to mutant SOD1 in a living system. To confirm this assumption, immunoprecipitation using monoclonal antibodies can be done on tissue sample homogenates from animals expressing SOD1 and treated with EGCG. The association of EGCG with SOD1 can then be determined by mass spectroscopy. Lastly, an alternative study using animal models is to examine the effect of EGCG on other SOD1 mutations, specifically those that lead to dimer destabilization. To date, cellular and mouse studies that examine the effect of EGCG in a SOD1-ALS system have only looked at the $\beta$-barrel mutation of G93A-SOD1, it would be interesting to see if mutations that result in dimer destabilization had a stronger response to EGCG. This would help to determine whether EGCG is more effective with different SOD1 mutations.

EGCG has demonstrated general anti-aggregation capabilities towards numerous proteins and therefore it would be rational to test the effect of this compound with other ALS causing proteins, including TDP-43 aggregates and C9ORF72 aggregates. Whether the reduction of aggregation would be effective in the disease course is unknown, but it could provide important information about the disease mechanism. For instance, if the disease is caused by toxic aggregate species and EGCG is able to eliminate aggregation then there should be a corresponding change in the disease course. However, if the disease is caused by the mislocalization of protein and loss of function of the protein in certain cellular compartments, an examination of the effect of EGCG on the restoration of protein localization and whether it effects the disease course could be insightful.

In addition to EGCG, other compounds in the initial \textit{in silico} drug screen that were not examined could demonstrate potential therapeutic value. For instance, the results of the \textit{in silico} drug screen predicted d-gallocatechol (EGC) would bind to SOD1 with stronger affinity than EGCG. This compound is particularly interesting because while it has a flavan group in
common with EGCG, it lacks the gallate moiety, which is believed to be responsible for its anti-aggregation capabilities. It is possible that EGC could stabilize the SOD1 dimer using a different mechanism that might be more specific towards SOD1 and it could potentially be better suited in stabilizing SOD1 than EGCG. Additionally, compounds like quercitrin could not effectively be examined with our assays, but were predicted to bind to SOD1 with strong affinity. Further investigation of these compounds using NMR and cellular models would prove valuable in testing their potential in SOD1-ALS therapeutics.

Lastly, I examined the effect of EGCG on the dimer interface mutation, A4V-SOD1. Other mutations located in different areas of the protein should also be investigated. Furthermore, other small molecule compounds that tested negative using ANS fluorescence and the A4V-SOD1 mutation should be screened against other SOD1 mutations to see whether they are more effective in stabilizing non-dimer interface mutations.

**Conclusion**

SOD1 is a widely studied protein with hundreds of point mutations scattered throughout its sequence that lead to ALS. In order to investigate the role of SOD1 in ALS, purification of large amounts of properly folded and metallated protein is required. Here, we introduce a novel SOD1 fusion protein that optimizes various aspects of the expression and purification process of this protein. In this construct, SOD1 is linked to a venus yellow fluorescent protein to increase its solubility, a his-tag for cheap, efficient, and easy purification, and lastly to a TEV cleavage site for a simple method to remove the fusion tag from SOD1. The final purified product demonstrated normal dismutase activity and proper metallation, making it suitable for future experiments. In addition to wildtype protein, a number of SOD1 mutant fusion proteins were constructed and purified in a similar fashion.
Mutant SOD1 has demonstrated itself to be a stable protein that is prone to aggregation. We hypothesize that mutations in SOD1 lead to an increased susceptibility of the protein to denaturing stress, causing it to misfold and aggregate more readily than wildtype SOD1. Additionally, because of SOD1’s role as an anti-oxidant, we speculate that oxidative stress is the denaturant driving misfolding. Our lab has demonstrated that in response to hydrogen peroxide treatment, catalytic histidines become oxidatively modified, leading to reduced metal binding, protein misfolding, and aggregation. I used this oxidation model to test several hits of an in silico drug screen, where I looked for compounds that reduced misfolding of denaturation-stressed mutant SOD1. The flavonoid compound of EGCG demonstrated that it could prevent misfolding measured by ANS fluorescence and aggregation measured using right-angle light scattering in a manner that was independent of its anti-oxidant function. This is the first report to indicate EGCG’s anti-aggregation properties in an ALS model. The mechanism of action of EGCG is believed to occur through its stabilization of the dimer interface and our preliminary results looking at dimer dissociation demonstrated that mutant SOD1 in the presence of EGCG resulted in a reduction in the rate of dimer dissociation in response to a chemical denaturant. These results implicate that EGCG prevents mutant SOD1 misfolding and aggregation through its ability to stabilize the dimer of mutant SOD1 and alludes to potential therapeutic value in ALS.
REFERENCES


