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# CuATSM Protects Against the in Vitro Cytotoxicity of Wild-Type-Like Copper-Zinc Superoxide Dismutase Mutants but not Mutants That Disrupt Metal Binding

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## Publication Details

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**Abstract**

Mutations in the SOD1 gene are associated with some forms of familial amyotrophic lateral sclerosis (fALS). There are more than 150 different mutations in the SOD1 gene that have various effects on the copper-zinc superoxide dismutase (SOD1) enzyme structure, including the loss of metal binding and a decrease in dimer affinity. The copper-based therapeutic CuATSM has been proven to be effective at rescuing neuronal cells from SOD1 mutant toxicity and has also increased the life expectancy of mice expressing the human transgenes SOD1G93A and SOD1G37R. Furthermore, CuATSM is currently the subject of a phase I/II clinical trial in Australia as a treatment for ALS. To determine if CuATSM protects against a broad variety of SOD1 mutations, we used a well-established cell culture model of SOD1-fALS. NSC-34 cells expressing SOD1-EGFP constructs were treated with CuATSM and examined by time-lapse microscopy. Our results show a concentration-dependent protection of cells expressing mutant SOD1A4V over the experimental time period. We tested the efficacy of CuATSM on 10 SOD1-fALS mutants and found that while protection was observed in cells expressing pathogenic wild-type-like mutants, cells expressing a truncation mutant or metal binding region mutants were not. We also show that CuATSM rescue is associated with an increase in human SOD1 activity and a decrease in the level of SOD1 aggregation in vitro. In conclusion, CuATSM has shown to be a promising therapeutic for SOD1-associated ALS; however, our in vitro results suggest that the protection afforded varies depending on the SOD1 variant, including negligible protection to mutants with deficient copper binding.

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# CuATSM protects against the *in vitro* cytotoxicity of wild type-like SOD1 mutants but not mutants that disrupt metal binding.

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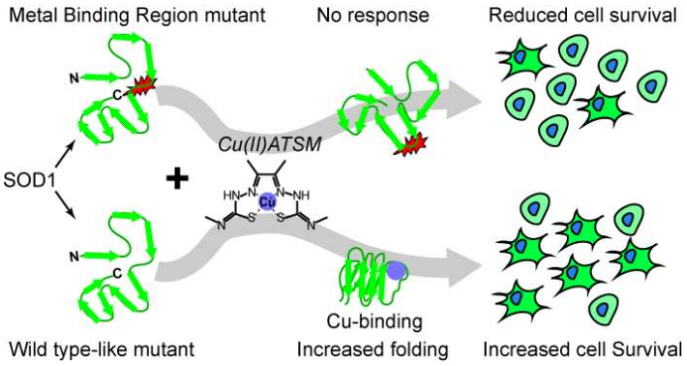
**Keywords: CuATSM, copper, protein aggregation, SOD1, ALS,**

**Abstract**

Mutations in the *SOD1* gene are associated with some forms of familial ALS (fALS). There are over 150 different mutations in the *SOD1* gene which cause various effects to the SOD1 enzyme structure, including loss of metal binding and a decrease in dimer affinity. The copper-based therapeutic CuATSM has been proven to be effective at rescuing neuronal cells from SOD1 mutant toxicity, and has also increased the life expectancy of mice expressing the human transgenes $SOD1^{G93A}$ and $SOD1^{G37R}$. Furthermore, CuATSM is currently the subject of a phase I/II clinical trial in Australia as a treatment for ALS. In order to determine if CuATSM protects against a broad variety of SOD1 mutations, we used a well-established cell culture model of SOD1-fALS. NSC-34 cells expressing SOD1-EGFP constructs were treated with CuATSM and examined by time lapse microscopy. Our results show a concentration dependent protection of cells expressing mutant $SOD1^{A4V}$ over the experimental time period. We tested the efficacy of CuATSM on ten SOD1-fALS mutants and found that while protection was observed in cells expressing pathogenic wild type-like mutants, cells expressing a truncation mutant or metal binding region mutants were not. We also show that CuATSM rescue is associated with an increase in human SOD1 activity and a decrease in SOD1 aggregation *in vitro*. In conclusion, CuATSM has shown to be a promising therapeutic for SOD1-associated ALS, however, our *in vitro* results suggest that the protection afforded varies depending on the SOD1 variant, including negligible protection to mutants with deficient copper binding.

## Graphical Abstract




## Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease and is the most common type of Motor Neuron Disease. ALS causes loss of muscle control and eventually leads to death due to respiratory failure, generally within 3-5 years after diagnosis[1]. These symptoms manifest due to the degeneration of upper and lower motor neurons in the motor cortex and spinal cord. The lifetime risk for ALS is ~1:300 to 1:400[2], and currently there are no effective treatments. Most cases of ALS are sporadic (sALS), however, approximately 10% are inherited. These familial cases (fALS) are linked to mostly missense mutations in over a dozen genes, including *SOD1*[3], *TARDBP*[4], *FUS*[5,6] and *CCNF*[7]. Furthermore, the most common genetic cause of ALS results from hexanucleotide repeats in non-coding regions of the *C9ORF72* gene[8,9].

*SOD1* was the first gene identified to contain mutations associated with fALS[3] and is the most widely studied of the currently known ALS-associated genes. Over 150 (mostly missense) mutations occurring throughout the protein sequence are currently thought to be fALS causative, with these mutations being responsible for ~20% of fALS cases[10]. The *SOD1* gene encodes an antioxidant enzyme, copper-zinc superoxide dismutase (SOD1), which functions as a cytosolic free radical scavenger. The protein has a homodimeric structure where monomers contain an intramolecular disulfide bond and also bind one copper (Cu) atom and one zinc (Zn) atom[11]. The maturation pathway of SOD1 is thought to proceed through Zn binding to the nascent polypeptide[12], followed by association with the copper chaperone for SOD1 (CCS), which delivers Cu and facilitates disulfide formation[13]. Cu is essential for its superoxide scavenging role as it reacts with oxygen free radicals in the following Cu-dependent reactions[14]:

1. $O_2^{\bullet-} + Cu^{II}ZnSOD \rightarrow O_2 + Cu^{I}ZnSOD$
2. $O_2^{\bullet-} + 2H^+ + Cu^{I}ZnSOD \rightarrow H_2O_2 + Cu^{II}ZnSOD$

It was initially thought that loss of enzymatic function was responsible for disease, however, this was determined not to be the case as many SOD1-fALS mutants retain enzymatic activity at near

wild type levels[15]. SOD1-fALS mutations are broadly designated into two classes; (i) wild type-like (WTL), which have similar dismutase activity levels to the wild type protein, and (ii) metal binding region (MBR) which are mutations occurring within the metal binding region of SOD1 that disrupt proper coordination of either Zn or Cu, resulting in decreased folding stability and enzymatic activity[11]. The existence of the WTL mutants suggests that pathology is caused by a toxic gain of function, most likely due to the misfolding and subsequent aggregation of mutant SOD1[11].

Growing evidence suggests that there is an association between protein aggregate formation and the development of ALS[16]. In SOD1-fALS it has been shown that affected neurons often have proteinaceous inclusions primarily composed of SOD1, and the aggregation propensity of SOD1 mutants correlate with cell death *in vitro*[17]. Also, the prion-like propagation of SOD1 misfolding and aggregation has been proposed to explain the progressive nature of the disease[18-21]. Aggregation of the SOD1 enzyme is generally associated with mutations that result in the destabilization of the structure from decreased metal binding[22], defective disulfide bond formation[23], increased dimer dissociation[24], and monomeric folding stability[25]. In addition, SOD1 is significantly metastable in motor neurons owing primarily to its extremely high expression[26] making it prone to aggregation, and it is expected that mutations act to exacerbate this situation.

Recently, the Cu-based small molecule Cu(II)ATSM (CuATSM) has been used experimentally to rescue ALS phenotypes in cell and mouse models[27-29]. Furthermore, human trials are currently underway as a potential treatment for ALS[26]. In separate experiments, CuATSM was shown to increase the survival of mice overexpressing the human SOD1 transgene with G37R [28,29] or G93A [27,30] mutations, both of which are WTL mutants. CuATSM was effective in protecting against SOD1-fALS mediated pathology in these experiments, however, since both mutations tested were WTL[11], whether CuATSM is still effective against MBR mutants is unknown. Therefore, using an established cell model of SOD1-related fALS[17,31,32], we set out to investigate whether CuATSM is protective across a range of ALS-associated SOD1 mutations. In the current work, ten SOD1-fALS mutations ($SOD1^{A4V}$, $SOD1^{G127X}$, $SOD1^{C6G}$, $SOD1^{G37R}$, $SOD1^{H46R}$, $SOD1^{E100G}$, $SOD1^{D90A}$, $SOD1^{G93A}$, $SOD1^{V148G}$ and $SOD1^{G85R}$) and wild type SOD1 ($SOD1^{WT}$) were used to examine mutant specific effects of CuATSM treatment (**Figure 1**). We found that of these mutants, cells expressing $SOD1^{H46R}$, $SOD1^{G127X}$ and $SOD1^{G85R}$, all which have disrupted Cu binding, were not protected by CuATSM. Further, we found that CuATSM significantly reduces the aggregation of pathogenic WTL mutants. These results are consistent with CuATSM delivering Cu to mutant SOD1 when Cu binding is not impaired, increasing protein stability, reducing aggregation and improving cell survival.

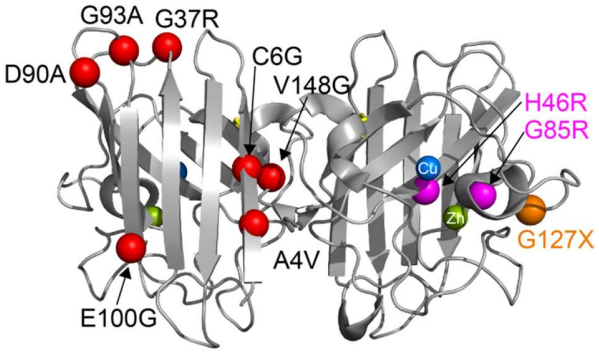



**Figure 1: SOD1 dimer with post-translational modifications and ALS-associated mutants.** (Left subunit) The location of the wild-type like (WTL) SOD1 mutants examined in this study (red spheres). (Right subunit) Cu (blue sphere) and Zn (green sphere) ions in their native binding sites. The metal-binding region (MBR) mutants H46R and G85R (magenta) shown close to the metal-binding pocket. The truncation mutant G127X is shown as an orange sphere. The disulfide bond is shown on both subunits (yellow sticks). PDB accession: 1HL5 [33].

## Results and Discussion

The therapeutic benefits of CuATSM have been experimentally proven in multiple models of SOD1-fALS[27-30], and independently verified in the SOD1-G93A mouse model[34], however, the exact mechanism of action in these models remains to be elucidated. Previous *in vivo* studies have used CuATSM on SOD1-fALS mice carrying transgenes for the WTL mutants $SOD1^{G37R}$ [29] and $SOD1^{G93A}$ [30], however, no experiments have been performed in models expressing SOD1 MBR mutants. Here, we have addressed this gap in knowledge by using CuATSM to treat a cell model of SOD1-fALS that allowed for rapid comparison of a broad range of mutants, finding that CuATSM alleviated pathology only for pathogenic WTL SOD1 mutants.

**CuATSM protects NSC-34 cells against mutant SOD1 toxicity in a dose dependent manner.** CuATSM has been shown to improve survival and locomotor function of SOD1 mice in a dose-dependent manner[29]. Here we sought to determine the effect of CuATSM on the survival of a well-established cell model; NSC-34 cells overexpressing human mutant SOD1-EGFP[17,31]. Using live-cell time-lapse microscopy we monitored the survival of NSC-34 cells transiently transfected with $SOD1^{WT}$-EGFP (**Figure 2A**), $SOD1^{A4V}$-EGFP (**Figure 2B**) or EGFP only control (**Figure 2C**) over a 90 h period in the presence of increasing concentrations (0 - 1 µM) of CuATSM. Consistent with what has been observed in transgenic SOD1 animal models[29], CuATSM increased the survival of mutant SOD1 expressing NSC-34 cells in a dose dependent manner. In the absence of CuATSM, $SOD1^{A4V}$-EGFP expression resulted in a significant loss of cells compared to those expressing $SOD1^{WT}$-EGFP. A concentration dependent increase in $SOD1^{A4V}$-EGFP cell numbers was observed post CuATSM treatment, except for at the 1 µM dose which resulted in a loss of cells relative to the 0.5 µM dose (**Figure 2D**). The protection afforded to $SOD1^{A4V}$-EGFP expressing cells by CuATSM was maximal at 0.5 µM. Treatment with 1 µM CuATSM was also toxic to cells expressing both $SOD1^{WT}$-EGFP and EGFP. Therefore, a CuATSM concentration of

0.5 μM was selected as the most appropriate dose to examine the toxicity associated with overexpression of mutant SOD1 in NSC-34 cells for subsequent experiments.

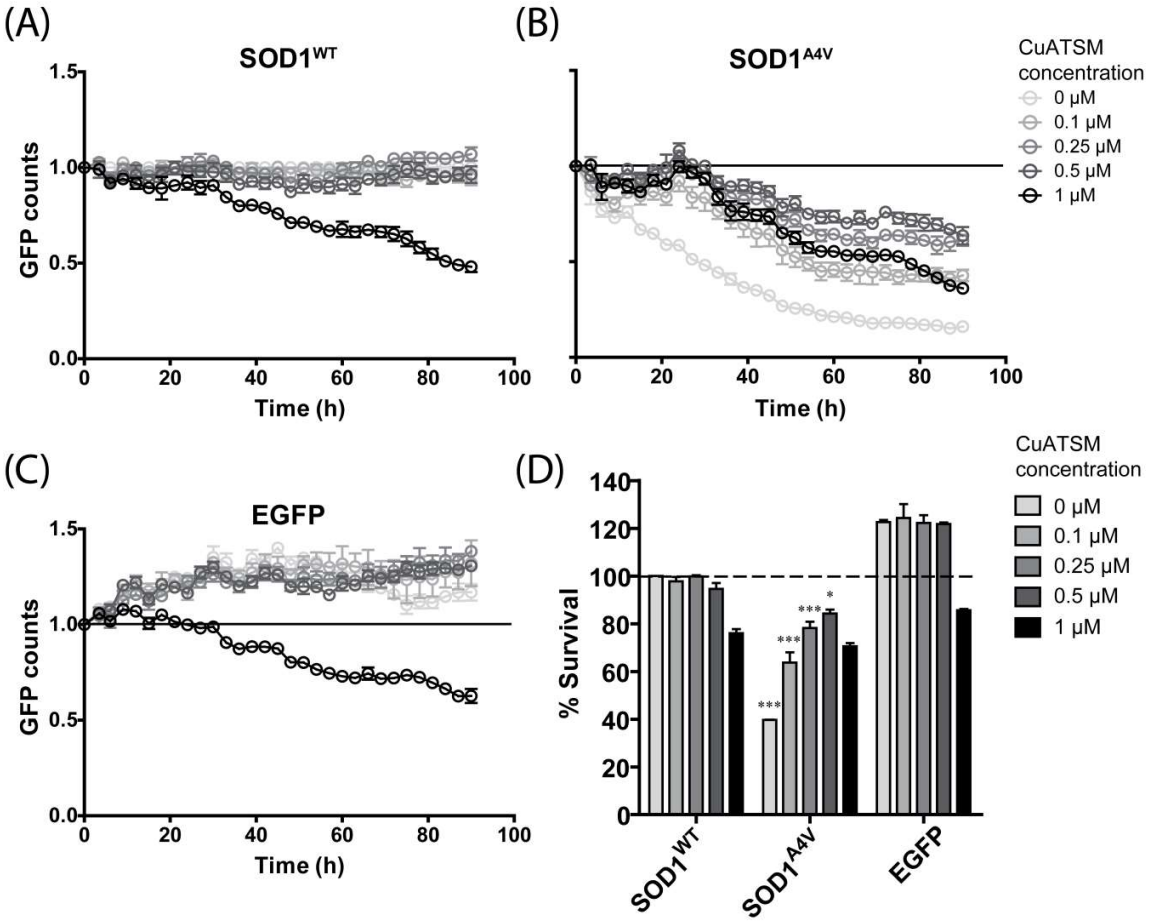



**Figure 2: The effects of CuATSM on the survival of NSC-34 cells expressing SOD1-EGFP.** NSC-34 cells expressing SOD1$^{WT}$ (A), SOD1$^{A4V}$ (B) and EGFP (C) were treated with 0-1μM CuATSM and images were acquired for 90 h every 3 h on a IncuCyte automated fluorescent microscope in the GFP channel. The number of GFP positive cells was determined at every time point and first normalized to the starting time point after which the value at each time point for mutants was normalized to the number of untreated SOD1$^{WT}$ cells at corresponding time points (indicated by the solid straight line in panels B and C). (D) The percentage survival of cells expressing SOD1$^{WT}$, SOD1$^{A4V}$ or EGFP in the presence of 0-1μM CuATSM was determined by measuring the area under the GFP count curves and normalizing to SOD1$^{WT}$ in the absence of CuATSM. Data shown are means ± SEM (n= 3). Each sample is an individually treated single microplate well. Two-way ANOVA with Bonferroni post-test was used to compare differences. Asterisks indicate a significant difference to SOD1$^{WT}$ (*$p<0.05$, ***$p < 0.001$).

**CuATSM does not protect cells expressing SOD1 mutations that disrupt metal binding.**
Although CuATSM has been previously shown to rescue SOD1 mouse models from overexpression of either SOD1$^{G37R}$ [28,29] or SOD1$^{G93A}$ [27-30], it has not been tested against other

SOD1 mutations, some of which are MBR mutations which affect Cu coordination. Having determined an effective dose of CuATSM to apply to our model, we transiently transfected NSC-34 cells with ten different mutant SOD1-EGFP constructs to determine the effect of CuATSM on a range of SOD1 mutants, including MBR mutations.

As we have shown previously, expression of human SOD1 mutants result in a loss of cell numbers relative to cells expressing $SOD1^{WT}$ [17]. An exception to this is the D90A mutation which is recessive in some Scandinavian families[35]. In fact, the pathogenic nature of D90A is contested, with some suggesting heterozygosity is enough to cause disease[36], while others have found that as many as 2.5% of the Northern Swedish population are carriers, suggesting D90A is not pathogenic[37]. Consistent with this is the fact that some D90A kindreds individuals without the mutation inherit ALS[38]. Our results are consistent with the latter in that expression of $SOD1^{D90A}$ did not produce a phenotype, and as such essentially acts as an additional WT control. In the case of cells expressing EGFP fused to $SOD1^{A4V}$, $SOD1^{C6G}$, $SOD1^{G37R}$, $SOD1^{E100G}$, $SOD1^{G93A}$, and $SOD1^{V148G}$, the addition of 0.5 μM CuATSM resulted in an increased number of SOD1-EGFP positive cells comparative to untreated cells at all time points (**Figure 3A**). After calculating the area under the curve across the entire time course, it is apparent that CuATSM treatment significantly improved transfected cell survival for these mutants (**Figure 3B**).

Cells transfected with MBR mutants $SOD1^{H46R}$ and $SOD1^{G85R}$ also exhibited a loss of transfected cells relative to $SOD1^{WT}$ over time. Similarly, the truncation mutant $SOD1^{G127X}$, that fails to fold and thus should not bind metals, also resulted in relative cell loss. However, addition of CuATSM did not alter the rate of cell loss (**Figure 3A**) and no significant differences in cell survival were observed with the addition of CuATSM in these mutants (**Figure 3B**). Cells expressing $SOD1^{D90A}$, EGFP or $SOD1^{WT}$ also were also unaffected by CuATSM but this was most likely due to the fact that expression of these constructs did not result in a significant loss of cells when left untreated.

Each pathogenic SOD1 mutant elicited a variable effect on relative NSC-34 cell survival (**Supplementary Figure 1**). In order to compare the amount of protection afforded to each mutant by CuATSM, a protection score (% protection) was calculated. The score represents the percentage protection CuATSM provides to cells compared to the vehicle control, regardless of differences in cell loss from $SOD1^{WT}$. This allows for direct comparisons across SOD1 variants. The analysis confirmed that cells expressing $SOD1^{G127X}$, $SOD1^{H46R}$, and $SOD1^{G85R}$ were not protected by CuATSM (**Figure 3C**). Cells expressing $SOD1^{WT,}$ $SOD1^{D90A}$ or EGFP were also not protected by CuATSM treatment. For all other SOD1 mutants, CuATSM had a significant protective effect on cell survival. Protection scores for these WTL mutants ranged from ~50% ($SOD1^{V148G}$) to ~85% ($SOD1^{G93A}$), suggesting CuATSM does not provide protection to all WTL mutants equally (**Figure 3C, Supplementary Table 1**). However, there was no relationship between protection score and associated clinical disease severity (**Supplementary Figure 2**).

The results reported here show that addition of CuATSM to cells expressing MBR SOD1-fALS mutants and truncation mutant $SOD1^{G127X}$ does not alleviate the toxicity of these mutant proteins. Furthermore, CuATSM does not affect cells expressing EGFP, $SOD1^{D90A}$, or $SOD1^{WT}$. However, similar to previously published data from mouse models[27-30], we show that CuATSM improves the survival of cells expressing pathogenic WTL SOD1 mutants.

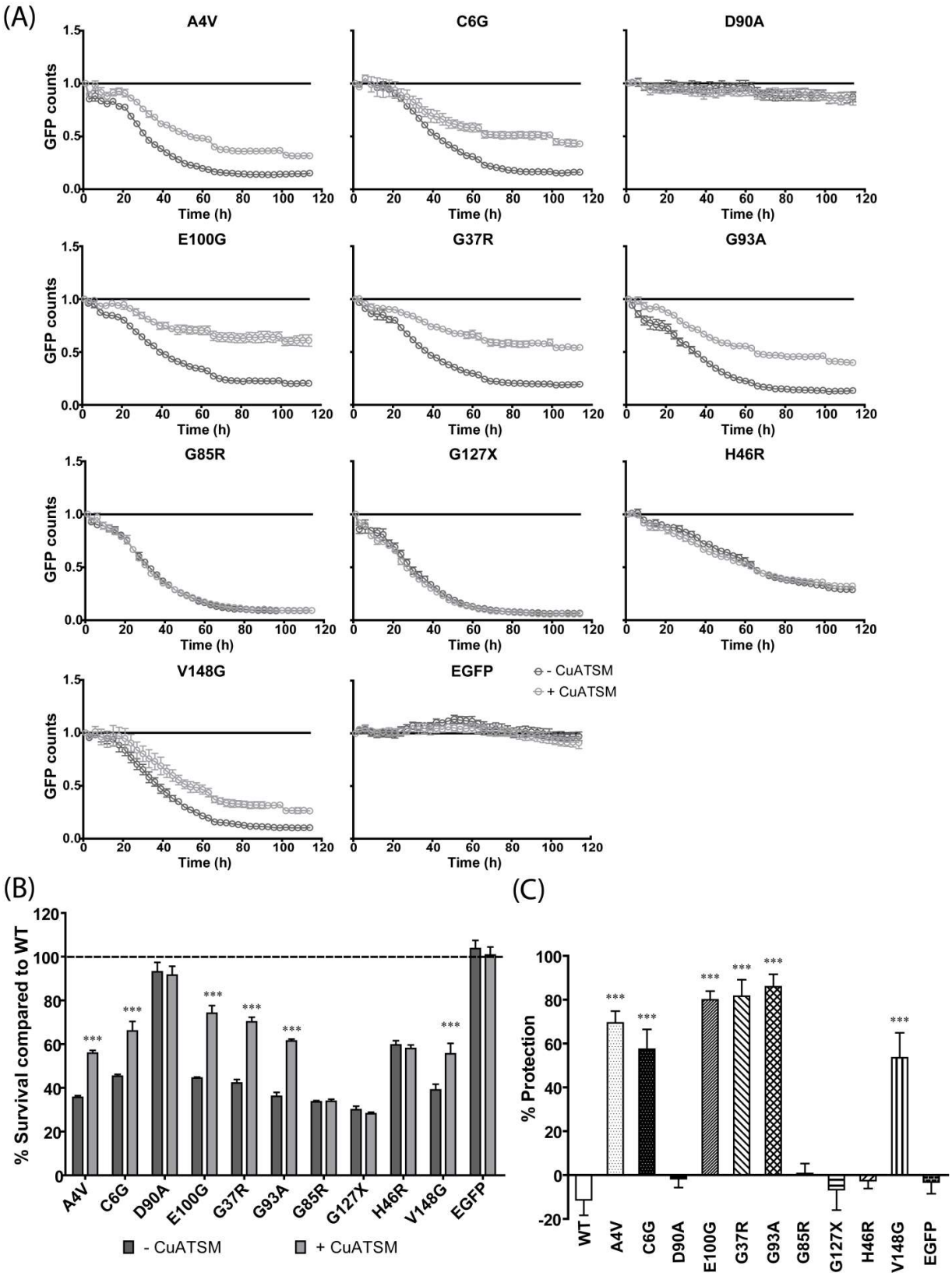

(A)
A4V
C6G
D90A
E100G
G37R
G93A
G85R
G127X
H46R
V148G
EGFP
GFP counts
Time (h)
- CuATSM
+ CuATSM
(B)
% Survival compared to WT
- CuATSM
+ CuATSM
(C)
% Protection
WT

**Figure 3: The effect of CuATSM on the toxicity of different SOD1 variants**. (A) NSC-34 cells were transfected with SOD1-EGFP constructs and GFP fluorescence was monitored over 120 h in the presence and absence of 0.5μM CuATSM. (B) Cell survival compared to cells expressing $SOD1^{WT}$ was determined by measuring the area under the curve for each SOD1 variant in the presence and absence of CuATSM over the 120 h time period. Data shown are means ± SEM (n= 3). Each sample is an individually treated, single microplate well. Data are representative of 3 independent experiments. Statistical significance was determined using two-way ANOVA with Bonferroni post-test (*** $p < 0.001$). (C) The protection provided to cells by CuATSM was determined by calculating the difference between each variant with and without CuATSM treatment as a percentage relative to untreated cells. Data shown are means ± SD (n= 3). Each sample is an individually treated, single microplate well. Data are representative of 3 independent experiments. Statistical significance was determined using one-way ANOVA with a Tukey's multiple comparison post-test. Asterisks indicate significant difference to $SOD1^{WT}$ (*** $p < 0.001$).

**CuATSM decreases the aggregation of SOD1 mutants in cultured cells.**

Although SOD1 is a small protein, it requires extensive processing and post-translational modification (PTM) to reach maturity[13], which includes Zn binding, Cu binding, and disulfide formation. These PTMs impart remarkable stability to the enzyme making it resistant to proteolytic digestion[39], thermal unfolding[40], and chemical denaturation[41]. Removal of these PTMs is considered necessary for its misfolding and aggregation[42]. Indeed, the thermal stability of mature mutant $SOD1^{G93A}$ is highly similar to $SOD1^{WT}$ [43], with significant differences between the stability of mutants only becoming apparent in the apo-state. Considering this, the availability of additional Cu imparted by CuATSM treatment, would be expected to aid CCS in its chaperone function[44] and increase maturation of the pool of intracellular SOD1 to a Cu bound form that is less susceptible to misfolding and aggregation.

To test whether CuATSM affects the aggregation of SOD1 in the NSC-34 cell model, cells expressing the various SOD1-EGFP constructs were treated with 0.5 μM CuATSM for 72 h before permeabilising cell membranes with saponin[45,46] to allow the diffusion of soluble protein out of the cell and facilitate examination of the insoluble EGFP signal remaining inside the cell[47] (**Figure 4A**). The amount of insoluble material (aggregates) remaining inside cells was then quantified as a proportion of transfected cells. We found that aggregation varied amongst SOD1 variants with as many as 22% of $SOD1^{A4V}$ expressing cells, and ~1% of $SOD1^{WT}$ and $SOD1^{D90A}$ expressing cells containing aggregates (**Figure 4B**). In cells where minimal aggregation occurred, specifically EGFP, $SOD1^{WT}$ and $SOD1^{D90A}$, CuATSM had no significant effect on aggregation. We also found that CuATSM did not significantly reduce the aggregation of MBR SOD1 mutants $SOD1^{H46R}$ and $SOD1^{G85R}$ (1.2 and 1.1 fold reduction in the percentage of cells containing aggregates compared to the vehicle control respectively; **Figure 4B**). Cells expressing the largely unstructured $SOD1^{G127X}$ also had a small but significant decrease in aggregation in the presence of CuATSM (~ 1.3 fold reduction). In contrast, cells expressing pathogenic WTL mutants all showed a significant reduction in cells containing aggregates (on average ~ 3 fold reduction, also see **Supplementary Table 1**) distinguishing them from the MBR mutants and controls (**Figure 4B**).

Although Cu deficiency is associated with increased misfolding of SOD1, it has previously been shown that treatment with CuATSM did not reduce the levels of misfolded SOD1 in $SOD1^{G37R}$ mice[28]. Interestingly, CuATSM has been shown to reduce the accumulation of abnormally phosphorylated and fragmented TDP-43 in the spinal cords of mice expressing low levels of mutant $SOD1^{G93A}$ [48]. This may be due to direct effects on SOD1 aggregation as SOD1 aggregates have been shown to induce TDP-43 mislocalisation[49]. Here, we show that CuATSM treatment reduces the aggregation of pathogenic WTL SOD1 mutants and has small but nonsignificant effects on the aggregation of MBR mutants and a small but significant effect on cells expressing $SOD1^{G127X}$. Similarly, CuATSM does not alter the minimal aggregation in cells expressing $SOD1^{D90A}$, EGFP or $SOD1^{WT}$. Our data suggests that the primary effect CuATSM has on aggregation is to supply Cu to mutant SOD1, thereby stabilizing its structure. The small reductions in aggregation observed with mutants unable to bind Cu suggests that CuATSM could also have additional indirect effects on aggregation by activating stress pathways via Nrf2[50], including the ubiquitin-proteasome system, but that this modest effect does not extend to cell viability. In the case of G127X, there is also a chance that since it retains the Cu-coordinating histidine residues (H46, H48, H63, and H120), Cu could be binding with low affinity. This could afford a slight increase in stability that plausibly translates to the minimal effect on aggregation observed here.

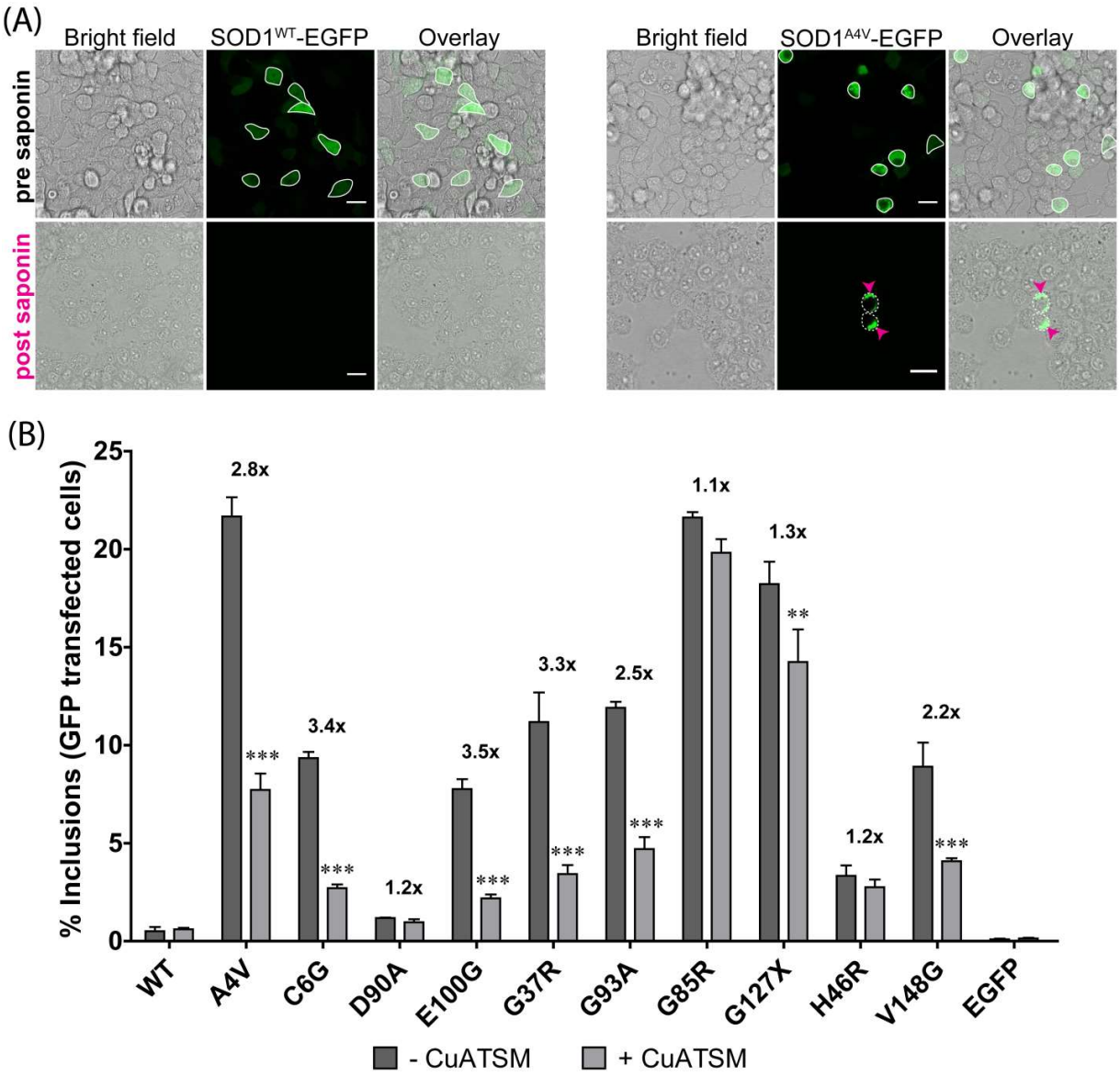



**Figure 4: CuATSM decreases the aggregation of WTL SOD1 mutants.** (A) NSC-34 cells transfected with SOD1$^{WT}$-EGFP or SOD1$^{A4V}$-EGFP were imaged by confocal microscopy pre- and post-saponin treatment to identify cells containing insoluble SOD1-EGFP aggregates. Scale bars are 20 µm and pink arrowheads highlight insoluble aggregates. (B) NSC-34 cells expressing SOD1-EGFP constructs were also imaged pre- and post-saponin treatment using an IncuCyte automated microscope to determine the proportion of transfected cells that contained SOD1-EGFP inclusions. Data shown are mean ± SEM (n = 3). Each sample is an individually treated, single microplate well. Data are representative of 3 independent experiments. The fold decrease in the number of cells containing aggregates seen with +CuATSM treatment (relative to the vehicle control, -CuATSM) is stated above each mutant. Statistical significance was determined by two-way ANOVA with a Bonferroni post-test (** $p < 0.01$, *** $p < 0.001$).

**CuATSM increases SOD1 levels and activity in WT-like mutants.**

Previous work demonstrates that administration of CuATSM results in the increase of human mutant SOD1 protein levels in mouse models of ALS[27,28]. The increase in SOD1 protein levels also corresponded to an increase in SOD1 activity[27] suggesting the additional protein was properly folded and metalated. To test whether SOD1 levels and activity were increased by CuATSM in the NSC-34 cell model used here, western blotting and in-gel zymography was performed on cell lysates from cells cultured in the absence or presence of CuATSM. Cells expressing $SOD1^{WT}$ and $SOD1^{A4V}$ and $SOD1^{H46R}$ were compared as examples of WTL and MBR mutants. In-gel zymography revealed that CuATSM increased the activity of human SOD1 in the case of cells expressing $SOD1^{WT}$ and $SOD1^{A4V}$ (**Figure 5, Supplementary Figure 3**). Similarly, CuATSM increased the activity of $SOD1^{D90A}$ and all pathogenic WTL mutants tested **(Supplementary Figure 3**). However, $SOD1^{A4V}$ remained predominantly monomeric regardless of treatment. This increase in activity was accompanied by increases in protein levels as determined by western blot (**Figure 6, Supplementary Figure 4**). In contrast, $SOD1^{H46R}$ was inactive in both the presence and absence of CuATSM (**Figure 5B**), and there was no difference in protein levels after treatment with CuATSM (**Figure 6B)**. In-gel zymography results also indicate that the MBR mutant $SOD1^{G85R}$ and truncation mutant $SOD1^{G127X}$ remain inactive regardless of CuATSM treatment (**Supplementary Figure 3**).

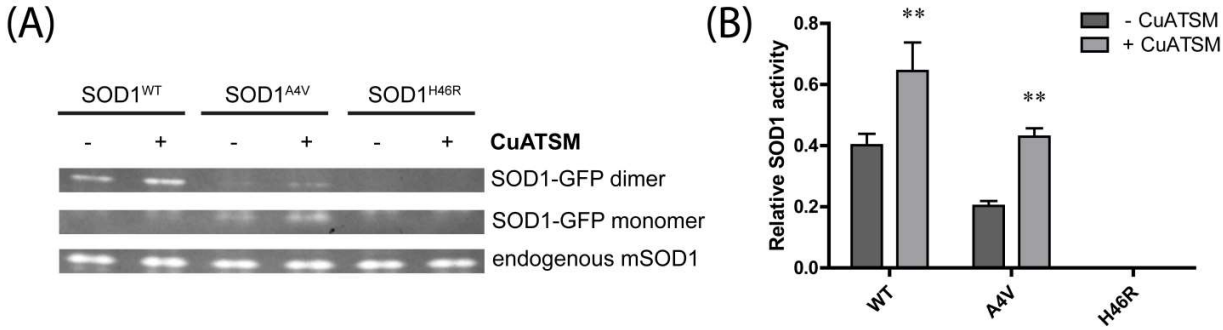



**Figure 5. CuATSM increases the activity of WT-like SOD1 mutants.** (A) Lysates from NSC-34 cells overexpressing $SOD1^{WT}$, $SOD1^{A4V}$ and $SOD1^{H46R}$ were separated on native 4-16% gradient bis-tris gels and SOD1 activity was determined by in-gel zymography. Equal loading was determined by measuring endogenous mouse SOD1 on the same gel. (B). Quantification of SOD1 activity expressed relative to endogenous mouse SOD1 levels. Data shown are mean ± SEM (n = 3). Data is from 3 independent experiments. Two-way ANOVA with Bonferroni post-test was used to compare differences between cell lysates cultured in the presence and absence of CuATSM (**$p<0.01$).

Large pools of Cu-deficient SOD1 have been detected in the spinal cords of $SOD1^{G37R}$ mice and treatment with CuATSM reduced this pool and subsequently increased the amount of fully metalated SOD1[28]. Here we show that CuATSM treatment increases the concentration of active enzyme and reduces the aggregation of SOD1 mutants in NSC-34 cells. These results suggest that, in our system, overexpression of pathogenic WTL SOD1 mutants exceeds basal Cu demands of the cell, creating a pool of Cu deficient SOD1 which misfolds and aggregates. Interestingly, overexpression of $SOD1^{WT}$ and $SOD1^{D90A}$ also results in a pool of Cu deficient SOD1 as treatment

with CuATSM increases human SOD1 activity in NSC-34 cells, however, this does not result in aggregation and toxicity. This is consistent with the notion that a misfolded conformer is required for toxicity and not Cu deficiency per se. While our results suggest that misfolding and aggregation are the common attributes of WTL and MBR mutants that are associated with toxicity, Cu deficiency could result in the misfolding of SOD1 and subsequent aggregation and toxicity. Indeed, as aggregated SOD1 propagates in a prion-like fashion[18-21], increasing the availability of Cu to cells could reduce the pool of misfolded SOD1 required for template driven aggregation and thus propagation.

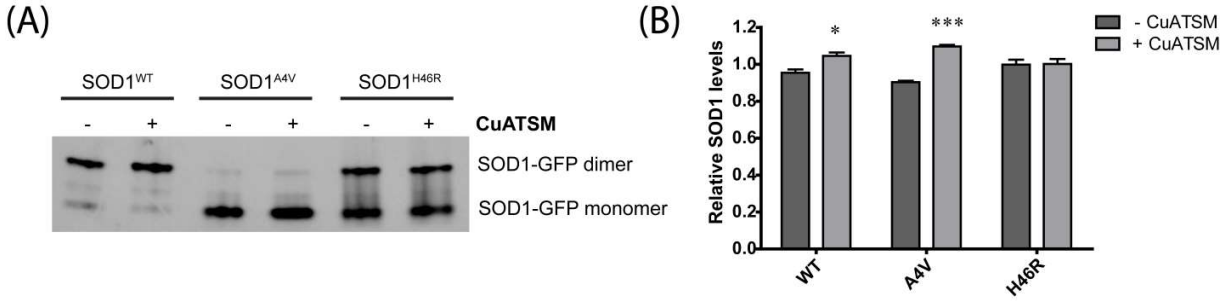



**Figure 6. CuATSM increases the levels of WT-like SOD1 mutants.** (A) Cell lysates from NSC-34 cells overexpressing $SOD1^{WT}$, $SOD1^{A4V}$ and $SOD1^{H46R}$ were separated on native 4-16% gradient bis-tris gels and SOD1-EGFP levels were determined by western blot analysis. (B) SOD1-EGFP levels were quantified from western blot analyses by densitometry and expressed relative to the average of the total EGFP signal for that variant. Data shown are mean ± SEM (n = 3). Data is from 3 independent experiments. Two-way ANOVA with Bonferroni post-test was used to compare differences between cell lysates cultured in the presence and absence of CuATSM (*$p<0.05$, ***$p<0.001$).

Copper homeostasis is an area of growing interest in SOD1-fALS research as Cu is found to be dysregulated in animal models expressing either WTL or MBR SOD1 mutants[51-54]. For example, Cu levels are found to be increased in affected neural tissues in SOD1-fALS mouse models including $SOD1^{G37R}$, $SOD1^{G93A}$, $SOD1^{WT}$, and even in mice expressing the MBR double mutant $SOD1^{H46R/H48Q}$ [51-54]. This suggests that Cu dysregulation in the central nervous system (CNS) is a common feature of SOD1 overexpression. The SOD1-associated Cu dysregulation in these models has been shown to affect other Cu-binding proteins such as cytochrome c oxidase[30] and ceruloplasmin[55]. Although this effect appears to be partially model-specific as cytochrome c oxidase activity was no different to controls in the latter study, this difference is likely due to the fact that the hCCS×$SOD1^{G93A}$ model is an extremely severe model of the disease. Furthermore, Cu dysregulation appears to occur early in SOD1 mouse models where changes to Cu levels in the CNS are detected as early as 30 days of age, well before symptom onset, in $SOD1^{G93A}$ mice[56]. Similarly, the $SOD1^{G37R}$ mouse model experiences a redistribution of Cu from the liver to the CNS at the embryonic stages of development, which is not seen in adult mice[57], suggesting changes to Cu regulation over time in SOD1 mouse models.

Considering the above work, there have been several proposals for the molecular mechanism(s) by which CuATSM attenuates the ALS phenotype in model systems. These proposals emphasize the role of CuATSM in restoring healthy SOD1 folding and function, or they emphasize the role of CuATSM in restoring Cu homeostasis to other proteins as well as SOD1. The interpretation is made difficult because of the concomitant effects of SOD1 misfolding and Cu dysregulation.

Evidence for a homeostatic role include research in which treating a SOD1$^{G93A}$ mouse model with CuATSM was shown to inhibit peroxynitrite activity[48]. Oxidative and nitrosative stress were also found to be reduced, although it is unclear if this resulted from the increased folding of SOD1. Also, the transgenic mouse line overexpressing human CCS and SOD1$^{G93A}$ (hCCS×SOD1$^{G93A}$) was found to have severe Cu dyshomeostasis, where treatment with CuATSM restored Cu binding to cytochrome c oxidase[30]. This model also presented the curious observation that levels of misfolded SOD1 did not decrease even though levels of Cu-bound SOD1 increased[27]. Other effects of CuATSM not related to the CNS have been reported, such as upregulation of antioxidant enzymes in human coronary artery smooth muscle cells and cardiac myocytes through activation of the transcription factor NF-E2 related factor 2 (Nrf2)[50]. Finally, several Parkinson's disease mouse models[58] were shown to have better outcomes when treated with CuATSM, consistent with the idea that misfolded non-metalated SOD1 is not required for its protective ability in neurodegeneration.

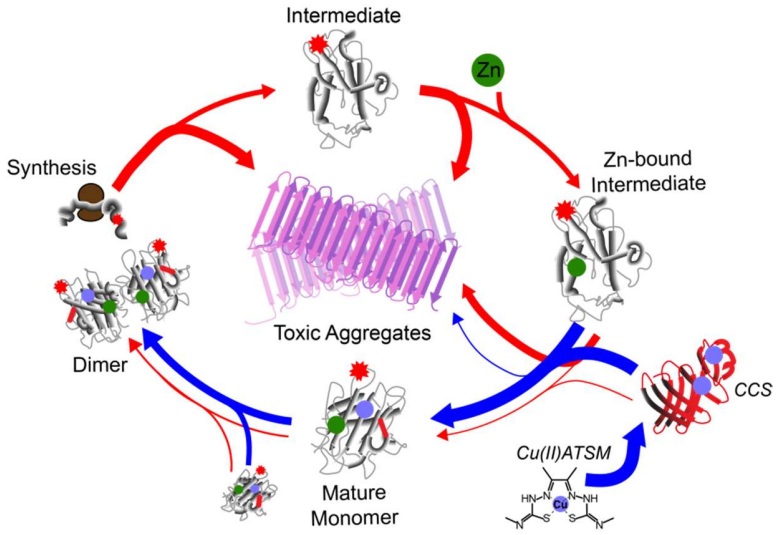



**Figure 7: A model of CuATSM rescue of mutant SOD1 pathology.** Red arrows show the folding and off-folding pathways for SOD1, whereas blue arrows show the contribution of CuATSM to these pathways. Arrow thickness suggests the probability of the pathways occurring. Following synthesis, mutant SOD1 (red start) folds into an intermediate state that is primed for Zn binding. Zn bound SOD1 associates with Cu-loaded CCS for transfer of Cu, leading to the formation of a mature SOD1 monomer that can form dimers. CuATSM results in a larger pool of Cu bound CCS which in turn results in greater transfer of Cu to SOD1, reducing the amount of SOD1 that enters an off-folding pathway at this point.

Our results, however, indicate distinct differences between MBR, a truncation mutation and pathogenic WTL mutants regarding the effect of CuATSM on the toxicity, aggregation, and maturation of SOD1 mutants in NSC-34 cells (**Figure 7**). This evidence supports a distinct role of CuATSM in alleviating SOD1 aggregation and toxicity only for mutants that are capable of being rescued. However, cultured cells do not effectively model the CNS, and it is likely that CuATSM has multiple modes of action in the complex environment of the brain, including on cell types other than neurons (e.g. astrocytes[59]). Future work examining the effect of CuATSM in SOD1 MBR mutant transgenic mouse lines will be needed to reconcile these data. Furthermore, the role of metals in neurodegeneration is still unclear. Most data linking aberrant metal interactions to neurodegeneration in humans comes from ecological and occupational studies[60]. It is possible that metal homeostasis, like proteostasis, declines or changes with age. More research into this important area is necessary to determine the exact links between neurodegeneration and metal homeostasis.

**Conclusions**

Our results suggest that CuATSM is protective against toxicity mediated by a range of pathogenic WTL SOD1 mutants but not in the case of MBR mutants or a truncation mutant in a cell culture system. These results may be relevant for the design of future clinical trials of CuATSM for MND patients, as the disease type (fALS or sALS), and specific SOD1 mutations linked to the fALS case, may both impact on the efficacy of the treatment.

## Methods

**Plasmids.** The pEGFP-N1 vectors containing human SOD1 sequences were generated as described[61].

**Cell culture and transfection.** Neuroblastoma × Spinal cord hybrid NSC-34 cells[62] were maintained in Dulbecco's Modified Eagles Medium/ Ham's Nutrient Mixture F12 (DMEM/F12) supplemented with 10% (v/v) foetal bovine serum (FBS, Bovogen Biologicals, Australia). Cells were maintained at 37 °C in a humidified incubator with 5% (v/v) atmospheric $CO_2$. Cells were transiently transfected using Lipofectamine 3000 (Invitrogen, USA) according to manufacturer's instructions with 0.5 μg DNA per well for a 24-well plate and 2.5 μg DNA per well for 6-well plates.

**CuATSM treatment.** Cu(II)ATSM (SYNthesis med chem, Parkville, VIC, Australia) was dissolved in DMSO to make a 10 mM stock. NSC-34 cells were treated with 0 - 1 μM CuATSM or DMSO vehicle control (diluted in culture medium) 24 h post transfection for up to 120 h.

**Time lapse imaging.** The viability of cells expressing SOD1 in the presence and absence of CuATSM was monitored over 120 h in an IncuCyte automated fluorescent microscope (Essen BioScience, USA) as described in[17]. NSC-34 cells were plated into 24-well plates at a confluency of ~ 65% and transfected with SOD1-EGFP constructs 24 h later. Cells were dissociated 24 h post transfection and replated in clear flat-bottomed 96-well plates at a confluency of 30% in phenol red free DMEM/F12 containing 10% FBS and 0 - 1 µM CuATSM or vehicle control (DMSO). Images were acquired every 3 h and analysed using a processing definition created to select GFP positive cells. The number of GFP positive cells was normalized to time zero before mutant SOD1 numbers were adjusted to $SOD1^{WT}$ values as previously reported[17]. To analyze differences between treatments, area under the curve analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad software, USA). The percent protection score was calculated from GFP count data normalized to t = 0 h, where the area under the curve (AUC) from t = 0 -72 h was calculated for both vehicle control and CuATSM treated cells. The follow equation was used:

$$\%\ Protection = 100\ \times \frac{(AUC\ CuATSM) - (AUC\ Vehicle)}{(AUC\ Vehicle)}$$

**Saponin treatment to detect insoluble aggregates.** NSC-34 cells expressing SOD1-EGFP were gently treated 72 h post transfection with 0.03% saponin in PBS to allow the diffusion of soluble intracellular SOD1-EGFP out of the cytoplasm to identify cells containing SOD1 aggregates[45,46]. Cells were imaged pre and post saponin treatment on a TCS SP5 confocal microscope (Leica, Germany) or by using the scan on demand function on the IncuCyte and the number of cells containing aggregates was determined as a proportion of GFP expressing cells.

**Cell lysis.** NSC-34 cells grown in 6-well plates and transfected with SOD1-EGFP were harvested 48 h post CuATSM treatment (72 h post transfection) with trypsin/EDTA (Gibco, USA). Cells were washed with PBS before being resuspended in RIPA buffer (50 mM TrisHCl pH 7.4, 1% (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1% TX-100, 0.1% SDS, 10mM NEM, 1 mM sodium orthovanadate, Halt™ Protease Inhibitor Cocktail (Thermo Scientific, USA)). Protein concentration was determined by BCA assay.

**SOD1 In-gel zymography.** SOD1 enzymatic activity was determined using in-gel zymography methods described previously[63]. Briefly, 30 µg of cell lysate or 100 ng of purified $SOD1^{WT}$ protein (prepared as previously described[17]) was loaded onto a NativePAGE™ 4-16% gradient bis-tris gel (Invitrogen, USA). Following electrophoresis (150 V, 3 h), gels were incubated in 5 mM nitrotetrazolium chloride for 15 min with gentle rocking protected from the light. Gels were then rinsed with water before incubation with 30 µM riboflavin and 10 mM tetramethylethylenediamine (TEMED) solution for 20 min with gentle rocking in the dark. Gels were subsequently developed by exposure to fluorescent light on a light box until a significant signal was detected (achromatic bands). Gels were imaged on the Amersham Imager 6600RGB and band intensity was quantified

using ImageJ 1.48v software[64]. SOD1-EGFP activity was subsequently determined after normalizing signal to purified SOD1 and endogenous mouse SOD1 levels.

**Western blotting.** Following in-gel zymography, proteins were transferred onto a nitrocellulose membrane (Pall Corporation, USA) using native transfer buffer (48 mM Tris, 39 mM glycine, pH 9.2 containing 0.04% (w/v) SDS). The membrane was then incubated in native transfer buffer containing 20% methanol before detecting GFP signal by fluorescence (excitation at 460nm) on the Amersham Imager 6600RGB. Equal loading of the gel was determined by staining membranes with Ponceau red.

**Statistical analyses.** Normally distributed data sets were analyzed for statistical significance via the following tests: one-way ANOVA with a Tukey's multiple comparison post-test when comparing the means of multiple data sets, and two-way ANOVA with Bonferroni post-test when assessing multiple variables.

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### Author Contributions

MRY performed experiments, analyzed data and wrote the initial manuscript. LM designed experiments, analyzed data and edited the manuscript. SP provided feedback on experimental results and edited the manuscript. NEF designed experiments, performed experiments, analyzed data and wrote and edited the manuscript. JJY conceived of the study, designed experiments, analyzed data and edited the manuscript.

### Notes

The authors do not declare any competing interests.

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# CuATSM protects against the *in vitro* cytotoxicity of wild type-like SOD1 mutants but not mutants that disrupt metal binding.

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## Supporting Information

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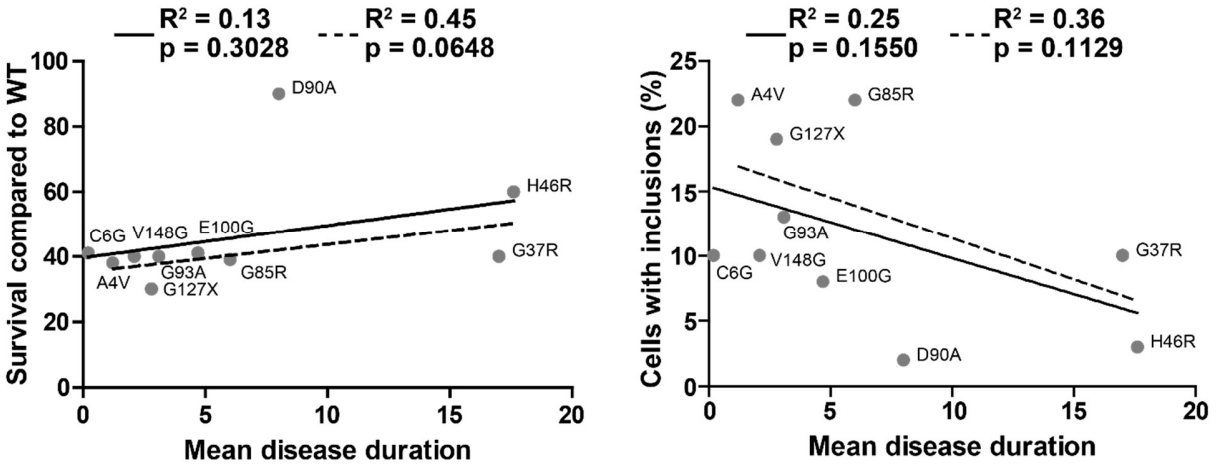
**Supplementary Table 1. Summary of SOD1 mutant characteristics and the effect of CuATSM measured in this paper**

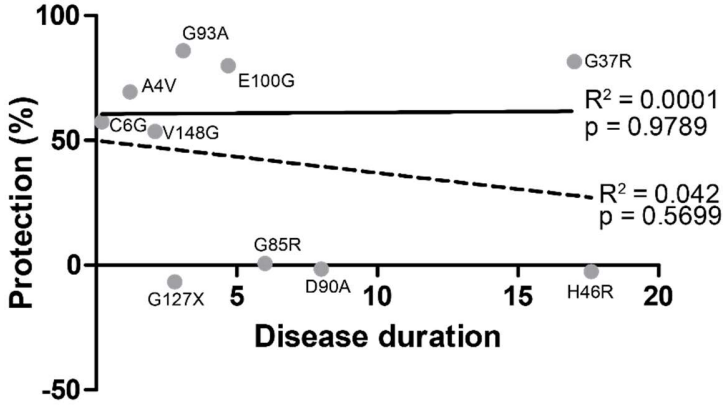
| SOD1 Mutant and class | Mutation Effects on stability (apo S-S state) compared to wild type | Mutation Effect on metal binding | Mutation Toxicity (% survival compared to WT) | CuATSM Protection Score (% protection) | Fold reduction in aggregation by CuATSM | Mean Disease Duration (years) |
|---|---|---|---|---|---|---|
| A4V<br>WTL | ↓↓↓- Monomer<br>↓↓↓↓ Dimer | ↓--- Zn<br>↓↓-- Cu | 35.7 | 69.4 | 2.8 | 1.2<br>(n=205) |
| C6G<br>WTL | (n/a) Monomer<br>(n/a) Dimer | (n/a) Zn<br>(n/a) Cu | 45.3 | 57.4 | 3.4 | 0.2<br>(n=2) |
| G37R<br>WTL | ↓↓-- Monomer<br>↓↓-- Dimer | ↓--- Zn<br>↓--- Cu | 42.2 | 81.6 | 3.3 | 17.0<br>(n=37) |
| H46R<br>MBR | ↑--- Monomer<br>---- Dimer | ↓--- Zn<br>↓↓↓↓ Cu | 59.7 | - 2.5 | 1.2 | 17.6<br>(n=49) |
| G85R<br>MBR | ↓↓↓- Monomer<br>↓↓↓- Dimer | ↓↓-- Zn<br>↓↓↓- Cu | 33.6 | 0.7 | 1.1 | 6.0<br>(n=11) |
| D90A<br>WTL | ↓--- Monomer<br>---- Dimer | ---- Zn<br>---- Cu | 93.0 | - 1.6 | 1.2 | 8.0*<br>(n=15) |
| G93A<br>WTL | ↓↓↓- Monomer<br>↓↓↓- Dimer | ---- Zn<br>---- Cu | 36.1 | 85.9 | 2.5 | 3.1<br>(n=16) |
| E100G<br>WTL | ↓↓-- Monomer<br>↓↓-- Dimer | ---- Zn<br>---- Cu | 44.5 | 80.0 | 3.5 | 4.7<br>(n=50) |
| G127X<br>truncation | ↓↓↓↓ Monomer<br>↓↓↓↓ Dimer | (n/a) Zn<br>(n/a) Cu | 30 | - 6.6 | 1.3 | 2.8<br>(n=3) |
| V148G<br>WTL | ↓↓↓- Monomer<br>↓↓↓- Dimer | (n/a) Zn<br>(n/a) Cu | 39.1 | 53.6 | 2.2 | 2.1<br>(n=11) |

References for SOD1 stabilities - [1-3]
References for SOD1 metalation - [4-6]
References for SOD1-fALS survival - [7,8]

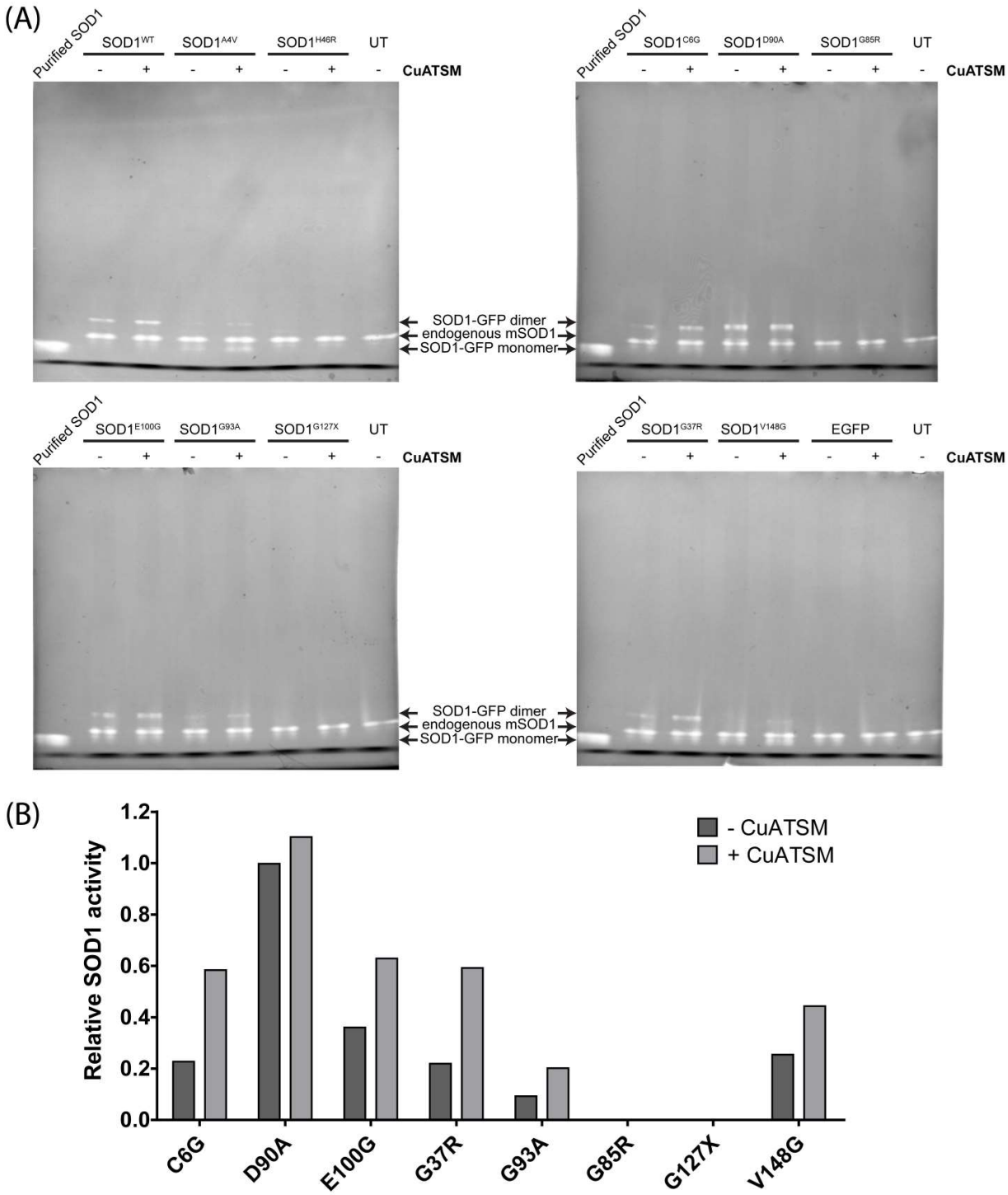
* the clinical severity of heterozygous D90A mutation is contested with some arguing its clinical relevance[9] and others that it is a common variant in some populations[10], while others argue another genetic element is responsible for ALS in D90A kindreds[11].



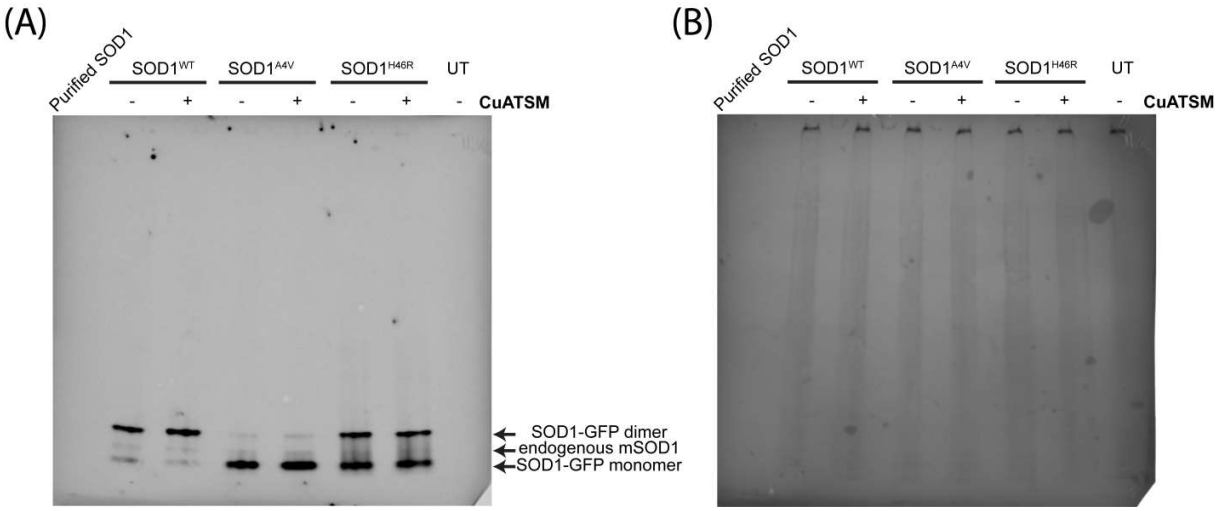

**Supplementary Figure 1: Correlations of patient mean disease duration against cell survival and inclusion formation.** The mean values of patient survival were taken from Wang *et al* (2008)[7] and plotted against the mean values from the data collected in this work for cell survival (left) and cells containing inclusions (right). Solid lines indicate the fit through all SOD1 mutant data points, whereas dotted lines indicate the removal of D90A and C6G from the fit. Data were fitted with least squares linear regression and the $R^2$ and p values are shown on each panel.




**Supplementary Figure 2: Patient disease duration does not correlate with CuATSM protection.** The mean values of patient survival were taken from Wang *et al* (2008)[7] and plotted against the percent protection values from the data collected in this work for only WT-like mutants (solid line) and all mutants (dotted line). Data were fitted with least squares linear regression and the $R^2$ and p values are shown next to each fit.




**Supplementary Figure 3:** (A) Cell lysates from NSC-34 cells overexpressing SOD1-GFP, as well as untransfected cells (UT) and purified $SOD1^{WT}$ protein were separated on native 4-16% gradient bis-tris gels and SOD1 activity was determined by in-gel zymography. (B) Levels of SOD1 activity were also determined for cell lysates expressing $SOD1^{C6G}$, $SOD1^{D90A}$, $SOD1^{E100G}$, $SOD1^{G37R}$, $SOD1^{G93A}$, $SOD1^{G85R}$, $SOD1^{G127X}$ and $SOD1^{V148G}$ by in-gel zymography. Data shown are for n = 1.




**Supplementary Figure 4:** (A) Representative western blot image of the effects of CuATSM treatment on SOD1 levels in cell lysates from NSC-34 cells overexpressing $SOD1^{WT}$, $SOD1^{A4V}$, or $SOD1^{H46R}$, as well as untransfected cells (UT) and purified $SOD1^{WT}$ protein separated on a native 4-16% gradient bis-tris gel. (B) Equal loading of the gel was determined by staining the membrane with ponceau red.

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