University of California

Santa Barbara

Cysteine sulfhydryls in tau display unusual hyperreactivity

A thesis submitted in partial satisfaction of the

requirements for the degree of Master of Science

in Biochemistry and Molecular Biology

by

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December 2020

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December 2020

Acknowledgements

This project would not have been possible without the guidance and expertise of Dr. John Lew and Dylan Peterson, as well as my labmates Malani Anand (who started this work), Aaron Kopperman, and Josh Dolinsky. Funding for this project was provided by the University of California, Santa Barbara in the form of a grant awarded to Dr. John Lew.

Abstract

Cysteine sulfhydryls in tau display unusual hyperreactivity

By

Yi-Li Chen Lam

One of the hallmarks of Alzheimer's disease is pathological tau aggregation. We previously reported that cinnamaldehyde, found in cinnamon, could inhibit tau aggregation *in vitro*, by reacting with one or both of the two cysteines in tau. In my thesis, I use spectrophotometric methods to show that both cysteines display hyperreactivity over model sulfhydryl compounds by as much as four fold. The hyperreactivity is specific to cysteines in tau as opposed to those in other sulfhydryl-containing cellular proteins. Cinnamaldehyde (CA) displays several characteristics that make it favorable as a possible inhibitor of aggregation *in vivo*: it is a water-soluble, brain-permeable molecule that is non-toxic and inexpensive. I now show in my thesis that the hyperreactivity of CA toward tau specifically, as described in this thesis, is an additional favorable property towards the goal of being a potential therapeutic

Introduction

The Significance of Alzheimer's Disease

Alzheimer's disease (AD) is a type of dementia characterized by an irreversible and steady decline in cognitive, functional, and behavioral ability. This neurodegenerative disease is one of the six leading causes of death in the United States (US)[1]. Healthcare expenses for Alzheimer's patients in their last five years of life can cost more than a quarter million dollars per person, 57% more than costs associated with diseases such as cancer and heart disease, which are the two leading causes of death in the US[2]. Yet despite its prevalence and costliness, no preventions or cures for this disease exist, only medications that treat symptoms[3]. Since the discovery of Alzheimer's more than a century ago, hundreds of trials have been performed and have yielded only five approved compounds, all of which merely slow the progression of the disease, but are ineffective in stopping or preventing it[4]. Without a breakthrough to prevent, cure or effectively slow AD, the number of people 65 years and older is expected to increase to 13.8 million and bankrupt the US healthcare system in only 30 years[5].

Tau and Alzheimer's Disease

More than three decades of basic science research have identified extracellular aggregates, called β-amyloid plaques, and intracellular twisted fibers, called tau tangles, as hallmarks of Alzheimer's disease[6]. Of these primary lesions, β-amyloid is the most common

target for therapeutics[7] since past evidence suggested that plaque formation was genetically linked to Alzheimer's and that it initiates the progression of this disease[6]. However, the failure of all amyloid burden clinical trials to date, as well as recent studies challenging the correlation of high β-amyloid plaque burden and neurodegeneration[8] both necessitate the exploration of alternative therapeutic approaches.

Neurofibrillary tangles, composed of tau protein which is mainly found in the axons of mature neurons, are another hallmark of Alzheimer's considered for targeting. Tau, in a healthy brain, is soluble in an aqueous environment and functions as a stabilizer, organizer, and facilitator of microtubules, which contribute to cell differentiation and polarization[9]. Its activity is regulated via phosphorylation at specific residues by kinases and phosphatases. Abnormal phosphorylation interferes with tau-microtubule binding and tubulin assembly, which may impact protein and neurotransmitter transport and therefore possibly cognitive function[10]. As the brain ages, tau proteins naturally begin to gradually aggregate. In patients with Alzhiemer's disease, however, aggregates of pathological tau are seen at a much higher concentration[9]. The resulting tangles correlate with neuronal loss and the severity of AD[11].

There are currently two anti-tau therapies undergoing human trials: LMTX is a tau aggregation inhibitor meant to alleviate neuronal damage, and LY3303560 is an antibody designed to capture and neutralize tau aggregates[7].

The Significance of Cinnamaldehyde

Since most small molecule chemical inhibitors don't make the drug pipeline due to their insolubility and inherent toxicity, our laboratory explored the aggregation-inhibition ability of several water-soluble compounds naturally found in edible plants. Investigating cinnamon and peanut skin extracts yielded evidence suggesting that the aggregation-inhibitory ability of these extracts was due to the polyphenols they contained[12,13]. Upon further examination, however, it was discovered that any observed inhibitory activity from these molecules was most likely due to non-specific, long-chain polyphenols in contaminating amounts (unpublished). However, after polyphenol removal using polyvinylpyrrolidone depletion, cinnamon extract still retained approximately 20% of its initial ability to inhibit tau aggregation. No reactivity was retained in the peanut skin extract, suggesting aggregate inhibition was specific to a molecule in the cinnamon extract and not in the peanut skin. HPLC analysis revealed an abundance of cinnamaldehyde (CA) in the cinnamon extract[12].

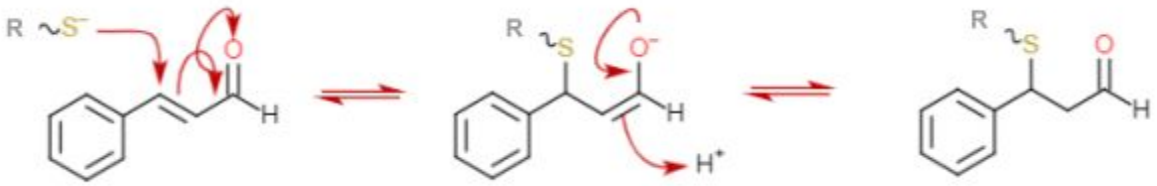

Figure 1. Mechanism for thia-Michael addition. Adapted from Figure 1 of *Nitro-fatty acid reaction with glutathione and cysteine - Kinetic analysis of thiol alkylation by a Michael addition reaction*[14]

The structure of CA (Fig. 2D) suggests its potential to react with free thiols, such as the two cysteines adjacent to each of tau's hexapeptide motifs (involved in pathogenic tau

aggregation). CA reacts with cysteines via a thia-Michael addition (Fig. 1), in which the cysteine's free thiol attacks the CA beta carbon, protonation at the CA alpha carbon occurs, and the CA aldehyde is re-formed[15]. Unlike Michael additions with carbon, oxygen, and nitrogen nucleophiles, thia-Michael additions are reversible[16]. While nucleophilic addition at the CA carbonyl carbon could be possible, our models, which assume the occurrence of a single and reversible reaction type (thia-Michael addition) between thiol-bearing species and CA, seem to adequately describe the behavior based on the closeness of fit to the collected data.

Previous evidence from our laboratory suggests that cinnamaldehyde is a tau aggregation inhibitor, and that it modifies tau's two cysteine residues to stoichiometry with no modifications to other residues (shown by mass spectrometry (ESI-MS))[13]. Additionally, whereas CA was able to inhibit aggregation with wild type tau187, CA failed to inhibit aggregation when both of tau187's cysteines were knocked out[13]. Such interaction strongly implies the existence of a mechanism of inhibition. In contrast, it could never be shown that polyphenols were able to bind to and modify tau. Here we show that tau is significantly more reactive with CA than the SKCGS peptide, strongly suggesting the contribution of additional features that enhance tau-cinnamaldehyde reactivity. Because of its ability to inhibit tau aggregation *in vitro*[13], as well as its water-solubility, brain accessibility[17], low toxicity, and low cost, we believe CA, or its derivatives, may prove to be a viable inhibitor of tau aggregation *in vivo*. In this study, we show significant specificity between CA and tau,

which makes CA an even more desirable candidate for development into a tau aggregation inhibitor relevant to Alzheimer's disease.

Materials and Methods

Tau Protein Constructs

All tau protein constructs bear polyhistidine tags for purification purposes and are stored at -80°C.

- Tau187: Residues 255-441 of human tau 441 (variant 2N4R); Expressed and purified by Dylan Peterson.
- C322S: Tau187 C322S; Expressed and purified by Joshua Dolinsky and Dylan Peterson.
- C291S: Tau187 C291S
- K19: Tau187 without repeat 2 or C-terminal domain (Fig. 2). Since K19 lacks tyrosine and tryptophan residues, the concentrations of reduced K19 samples were determined by DTNB post-reduction (see Cinnamaldehyde DTNB Assays: Reducing) using a 1:1 thiol-to-K19 ratio as a reference.
- S413C: Tau187 C291S C322S S413C; Expressed and purified by Dylan Peterson.
- 25-mer: The peptide, synthesized by GenScript USA Inc. (Piscataway, NJ), consists of the SKCGS peptide with 10 extra residues on either side which encompasses residues 310-335. It also features an acetylated N-terminus and an amidated C-terminus. The

concentration of each diluted sample was determined by DTNB assay. Solutions were stored at -20°C in water. (Order ID U8295FI020-1/PE8205)

Non-Tau Compounds/Samples

- SKCGS: The peptide, synthesized by GenScript USA Inc. (Piscataway, NJ), features an acetylated N-terminus and an amidated C-terminus. The concentration of each diluted sample was determined through calculation and compared with thiolate concentrations obtained through DTNB assay. Solutions were stored at -20°C in water. (Order ID U8131DG240-1)
- L-Glutathione (GSH): Classified as a model compound for this study, GSH was stored at 22°C in powder form. Fresh solutions were made with Nanopure water prior to each reaction timecourse and concentrations were confirmed via DTNB assay. (Alfa Aesar product #: J62166)
- 2-Mercaptoethanol (BME): Classified as a model compound for this study, BME was stored at 22°C in liquid form. Fresh dilutions were made with Nanopure water prior to each reaction and concentrations were confirmed via DTNB assay. (Fisher Biotech product #: BP176-100)
- Yeast Extract: Stored at -80°C. Prepared and purified by Dylan Peterson: *Saccharomyces cerevisiae* was lysed with a scoop of glass beads along with protease inhibitors and 625 uM DTT. After 5 cycles of alternating between 1 minute of vortexing and 1 minute of cooling on ice, the sample was drained from the microfuge tube into a 15 ml falcon tube

by spinning at 1500 x g for 2 minutes in a Sorvall X1, resulting in a pellet and supernatant. To clean the supernatant of residual debris after transfer, it was spun at top speed in a table top centrifuge for 10 minutes then filtered through a 5 ml G10 column to produce the desired yeast extract. Prior to reacting with cinnamaldehyde, each sample was pre-reduced with 1mM DTT and passed through a 1 ml G25-80 column (see Cinnamaldehyde DTNB Assays: Reducing). Sulfhydryl concentrations were determined with DTNB.

- Wild Type Mouse Brain Extract: Stored at -80°C. After removal and rinsing with homogenization buffer (50 mM Tris, 150 mM NaCl, 1 mM $NaVO_3$, 2 mM EGTA, 10 mM NaF, pH = 7.4, 0.5 mM PMSF, protease inhibitor cocktail (1 uM aprotinin, 1 uM pepstatin A, 1 mM leupeptin, 1 mM EDTA)), brains were submerged in 7 mL of homogenization buffer per gram of organ before homogenization and centrifugation at 15,000 x g for 20 minutes at 4°C. Brain supernatant was removed using a syringe to avoid disturbing any fat floating on top.

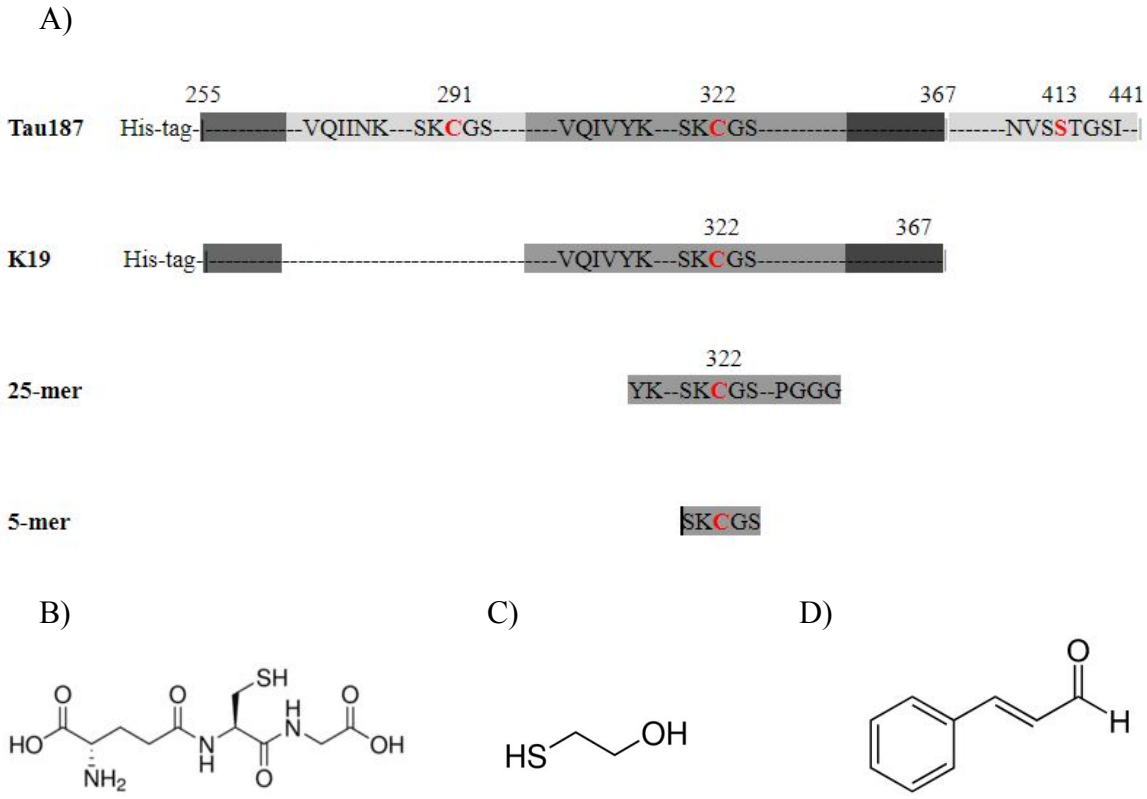

Figure 2. Panels A-C comprise of thiol-bearing nucleophiles used: A) Tau187: residues 255-442 of human tau isoform 2N4R. Cysteines 291 and 322, as well as serine 413, are indicated in bolded red. Hexapeptide motifs VQIINK and VQIVYK, established as essential for pathological tau aggregation, are also shown. Panel A is only for descriptive purposes and has not been drawn to scale. B) L-Glutathione (GSH). C) 2-Mercaptoethanol (BME). D) Cinnamaldehyde (CA).

Protein Expression

For each construct, the respective BL21 E. coli were grown in Luria Broth (LB) media and induced so that they over-expressed the desired protein. Under sterile conditions, a chunk of glycerol stock was used to inoculate 25 mL of Luria Broth per liter of bacteria desired. Kanamycin (Kan50) was added so its final concentration was 50 ug/mL. Then, with its tin-foil lid closed loosely to allow for oxygen circulation, the mixture was placed in a shaker that was set at 37°C at 27k rpm to produce an overnight culture. To grow a liter of BL21, 25 mL of overnight culture were added to 1 L of Luria Broth containing Kanamycin under sterile conditions and shaken at 37°C until it reached an OD600 reading of around 0.7. After

induction with 1 mL sterile IPTG per 1 L of LB, the bacterial culture was then shaken for another 2 hours under previous shaking conditions before the bacteria were harvested.

Purification: Ni-NTA Affinity Chromatography

After the BL21 E. coli were lysed, a 1 mL nickel resin column was used as the initial step in the protein purification process. Each protein construct had a hexa-his tag, allowing it to be immediately and selectively isolated from the rest of the bacterial lysate and proteases. After the resin was washed with buffers of low imidazole concentration to eliminate extraneous molecules, the resin was washed with a buffer of high imidazole concentration to elute the tau constructs. The high concentration of imidazole outcompetes the histidine-nickel coordination so that the tau constructs can be released.

Purification: Sepharose Ion-Exchange

To further isolate the tau constructs, the most concentrated Ni-NTA fractions were pooled and purified using a 5 mL sepharose (SP) ion-exchange column. After the protein was loaded onto the column using a buffer of low salt concentration, a salt gradient of progressively increasing NaCl content was employed to elute the tau constructs into approximately 50 fractions. By using a salt gradient, certain large-molecular-weight proteins that were previously unable to be separated were able to be excluded from the pooled SP fractions.

Purification: Gel Filtration Size Exclusion

In the last major step of protein purification, the pooled SP fractions were first concentrated then loaded onto a 5 mL G10 gel filtration column to remove DTT and salt from the

previous column buffers. Following elution, the fractions were pooled and then concentrated once more. Protein concentration was determined with the Nanodrop 2000C A280 function (1 cm pathlength) prior to storage at -80 degrees Celsius.

Reducing:

To pre-reduce, each tau construct was incubated in 1mM DTT for 1 hour at room temperature. Model compounds 2-mercaptoethanol and GSH, as well as SKCGS, were already in reduced form. Yeast extract samples were also subjected to sulfhydryl pre-reduction.

Desalting:

To remove DTT from the protein post-incubation, the pre-reduced sample was filtered through a 1ml column of G25-80 gel filtration resin and eluted with 1X reaction buffer (either MOPS buffer or Intracellular MOPS buffer, depending on experiment) into 10 fractions of ~120 ul.

DTNB Assays:

At each timepoint, 40 ul of the reaction mixture was removed and mixed with an equivalent volume of DTNB dissolved in room temperature 10 mM monobasic KH_2PO_4 buffer at pH 8.0 for a final DTNB concentration of 1 mM. The concentrations of free sulfhydryls remaining in the reaction mixture was determined by A412 using a Shimadzu UV-1601 UV-Visible Spectrophotometer. The spectrophotometer was "zeroed" with Nanopure water, then the A412 of a "blank" sample (40 ul of 2mM DTNB plus 40 ul of 1X reaction buffer) was

measured and subtracted from the A412 of each sample to account for background absorbance from excess DTNB.

- MOPS buffer: The 10X buffer (150 mM MOPS, 500 mM NaCl, pH = 7.0 unless otherwise stated) was stored at room temperature.
- Intracellular MOPS buffer: The 10X buffer (150 mM MOPS, 1500 mM KCl, 100 mM NaCl, 5 mM $MgCl_2$, 1000 uM EDTA, pH = 7.2 unless otherwise stated) was stored at room temperature.
- DTNB: The 2 mM DTNB solution was made with 10 mM phosphate buffer (10 mM monobasic K_2HPO_4, pH = 8.0) and stored at -20°C. The A412 of the 1 mM DTNB solution is typically between 0.14 and 0.18. The extinction coefficient used was 11,400. From SIGMA (Product #: D-8130).
- Cinnamaldehyde (CA): The stock solution was stored at 4°C in liquid form. Samples were made fresh prior to each experiment by diluting stock solution 100-fold with 70% ethanol before further dilutions were made with Nanopure water. The extinction coefficient used was 25,000. Concentrations were confirmed via the Nanodrop 2000C UV-Vis function (1 mm pathlength) and were matched with the calculated stock bottle molarity. From Acros Organics (Product #: 110355000).

All reactions were done at room temperature (22°C) unless stated otherwise.

Modeling and Calculations

Curve fitting was done using a second order, reversible model in the Simbiology application from MatLab. MatLab plot and ODE solver functions were first used to find manual estimates of fit parameters with a series of second order differential equations. These, along

with experimentally determined initial CA and thiol concentrations, were input into the Simbiology application as initial guesses to generate association and dissociation rate constant parameters at a confidence level of 68% using the Gaussian method. The sample standard deviation formula used to calculate Table 1 standard deviation values is the following:

$$\sigma = \sqrt{\frac{\sum(x_i - \mu)^2}{N}}$$

Determination of Brain Extract Free Thiol Concentration:

The ratio of thiol concentration to protein concentration in the peak fraction of brain extract was determined after reduction with DTT and desalting. The concentration of thiols was then re-adjusted according to the total protein concentration in the extract, which was determined via A280 prior to the reduction process. Finally, this value was multiplied by 8 due to the approximately 8-fold dilution with homogenization buffer during the brain homogenization process.

Results

We previously showed that cinnamaldehyde could inhibit tau aggregation *in vitro*. We also showed by electrospray mass spectrometry (ESI-MS) that cinnamaldehyde (CA) reacts with tau *in vitro* through its cysteines (291 and 322) and nowhere else[12]. CA's inhibitory effects on aggregate formation *in vitro* seem to be due to a modification of these cysteines through a

reversible Michael addition[13]. To investigate the reactivity of these cysteines in greater detail, a series of kinetics experiments were performed which yielded the microscopic association and dissociation rate constants of reactions between CA and several tau constructs and model sulfhydryl compounds. This characterization of the kinetics of reaction between cinnamaldehyde and various thiol types allowed for a quantitative comparison of reactivity.

To characterize the kinetics of each cysteine individually and determine each sulfhydryl's contribution to overall tau reactivity, we made use of site-directed mutants. Two versions of tau were constructed, each with one or the other cysteine (291 or 322) mutated to a serine to eliminate sulfhydryl reactivity at that position. The C291S construct of tau has an intact cysteine at position 322 and a serine mutation at position 291. C322S has an intact cysteine at position 291 and its 322 cysteine has been replaced with a serine. Most of the characterization in this project was done on cysteine 322, using tau mutant C291S. As described in Figure 3B, either C322S or C291S was reacted with CA. The reaction was monitored until a majority of the its amplitude had been observed, meaning that a majority of the reaction had occurred. The data was then modeled according to classic second order kinetics in order to obtain the best-fit association and dissociation rate constants (Table 1). Since CA reacts with tau sulfhydryls in a reversible manner (see introduction), both association and dissociation constants were required to accurately describe the CA-tau reaction.

When comparing C322S to C291S, cysteine 322 (C291S) consistently reacted with nearly identical kinetics to cysteine 291 in C322S (Table 1). To test if the 2 cysteines display synergy, we tested tau187 (Fig. 3A). Since tau187 contains both cysteines and CA reacts

with tau187 with the same average kinetics, the cysteines at positions 291 and 322 do not appear to interact.

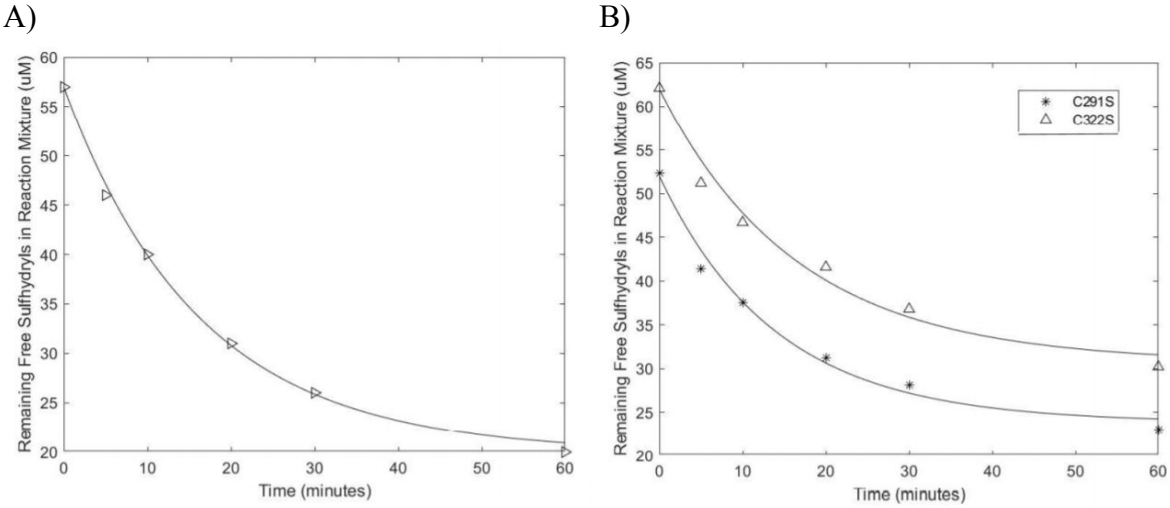

Figure 3. Comparison of Tau187 (wild-type) with C322S and C291S tau mutants at room temperature (22°C) and in MOPS Buffer: A) 57 uM Tau187 and 610 uM cinnamaldehyde (CA) were reacted at room temperature. **B)** 62 uM C322S was reacted with 710 uM CA at room temperature. 52 uM C291S was reacted with 710 uM at room temperature in parallel with the C322S reaction. The final concentration of MOPS Buffer (pH 7.0) for all three reactions was 15 mM MOPS and 50 mM NaCl. Remaining sulfhydryl concentrations and curve fitting parameters were determined as described in the Materials and Methods section.

In the tau protein, both cysteines 291 and 322 are sandwiched within the same five-residue sequence, SKCGS. We then asked if a peptide corresponding to this repeat would possess similar reactivity with CA. Surprisingly, it did not: the association rate constant of tau was three times larger than that of SKCGS and the dissociation rate constant of tau was two times larger than that of SKCGS. Since tau is an unfolded protein, it was unclear what kind of structural determinants contributed to its thiol hyperreactivity.

Having identified that CA does not react as fast with SKCGS as it does with tau, we wondered if it was SKCGS that was reacting slower than normal or if it was tau that was reacting faster than normal. We therefore compared the reactivity of CA and SKCGS with

that of the model compounds glutathione (GSH) and 2-mercaptoethanol (BME) (Fig. 4). The resulting association and dissociation rate constants (Table 1) show that SKCGS reacts more similarly to the model compounds than to tau, suggesting that the cysteines in tau are abnormally hyperreactive by as much as four-fold.

We then asked if the observed sulfhydryl hyperreactivity was unique to the tau protein or if this phenomenon was displayed by other proteins. This is important, because if the cysteines in tau are specifically hyperreactive compared to other cytosolic protein sulfhydryls, tau present in the complicated cytosolic extract of neurons may display significant specificity for reaction with exogenous CA *in vivo*.

To test this, the ideal experiment would have been to determine the average kinetic reactivity of all protein sulfhydryls in a bona fide mammalian neuronal extract in the absence of tau protein. This however was not possible since we were unable to obtain a mammalian, neuronal, cytosolic extract devoid of endogenous tau protein. We did not have access to neurons from genetically engineered tau knock-out mice so we therefore employed an extract corresponding to the cytosolic fraction of yeast. Since both extracts had many non-specific thiol-bearing proteins, we assumed that the extracts from both yeast and mammalian neurons would behave similarly in terms of sulfhydryl reactivity.

The yeast cytosolic extract was reduced with DTT, desalted, and reacted with DTNB according to the Materials and Methods. To determine the amount of extract in each reaction, we determined the molar ratio of thiol to protein using DTNB and A280 assays, respectively. We then measured the kinetics of reaction with CA and found that the average sulfhydryl reactivity in yeast extract was similar to that of SKCGS (Table 1). This sulfhydryl reactivity

corresponds to the extract's protein sulfhydryls, specifically, since GSH would be expected to have been removed during desalting (see Materials and Methods).

Table 1. A Comparison of Thiol Kinetics Parameters

Species	Replicates	Association Rate Constant (e-05 uM^-1 min^-1)	Association Rate Constant Standard Deviation (e-05 uM^-1 min^-1)	Dissociation Rate Constant (e-02 uM^-1 min^-1)	Dissociation Rate Constant Standard Deviation (e-02 uM^-1 min^-1)	Equilibrium Constant
Tau187	3	6.04	0.98	2.26	0.73	375
C322S	4	5.25	0.70	2.33	0.54	445
C291S	15	6.87	1.02	2.47	0.51	360
K19	4	5.88	1.34	2.12	0.61	361
S413C (C291S C322S)	3	4.64	0.39	1.76	0.06	380
25-mer	7	4.19	1.10	1.70	0.82	405
SKCGS	6	1.80	0.29	1.46	0.21	812
Yeast Extract	5	1.62	0.42	1.65	0.30	1017
GSH	3	0.75	0.13	0.43	0.12	574
BME	4	0.48	0.03	0.28	0.04	578

Each reaction was done at room temperature (22°C) in 1X MOPS buffer (15 mM MOPS, 50 mM NaCl, pH = 7.0 - 7.2) with 10 times the concentration of cinnamaldehyde (CA) as the initial concentration of free sulfhydryls (determined by DTNB and confirmed by the ratio of thiols to protein concentration from A280). Average values for association and dissociation constants were calculated with rate constants from experimental replicates with the best fitting data. Standard deviations were calculated using the sample standard deviation formula (see Materials and Methods). Equilibrium dissociation constants were calculated by dividing each average dissociation rate constant by its corresponding association rate constant.

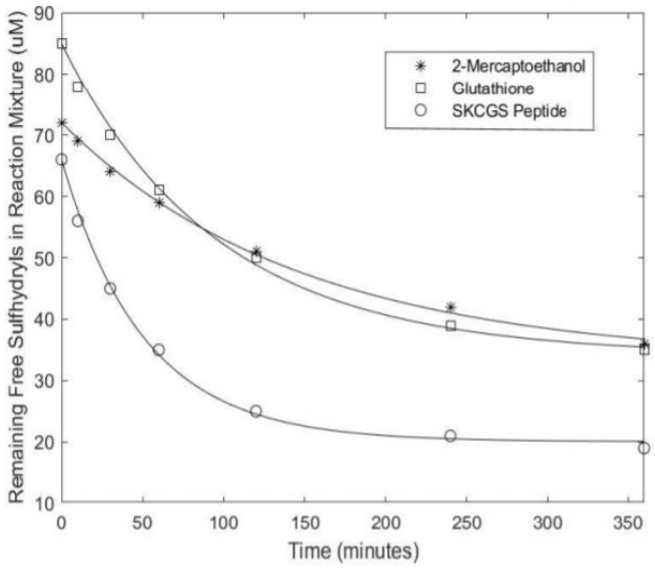

Figure 4. Comparison of SKCGS Peptide with Model Compounds GSH and BME at room temperature (22°C) and in MOPS Buffer: 66 uM SKCGS, 85 uM L-glutathione, and 72 uM 2-mercaptoethanol were each reacted with 700 uM CA in MOPS buffer at 22°C and monitored for 6 hours. The final concentrations of MOPS Buffer (pH 7.2) were 15 mM MOPS and 50 mM NaCl. Remaining sulfhydryl concentrations and curve fitting parameters were determined as described in the Materials and Methods section.

Because our data suggests an existence of CA-sulfhydryl hyperreactivity that is unique to tau, we attempted to determine the cause of this behavior. Our approach to identifying the basis of hyperreactivity was to examine the reactivity of tau constructs in which various regions were removed. We first tested K19, a version of tau missing both tau187's C-terminal domain (residues 368-441) and Repeat 2 (residues 274-304). A reaction between K19 and CA was monitored over the course of an hour, using C291S a control. The resulting association and dissociation constants suggest K19 reacts similarly to C291S (Table 1).

Since K19, with 106 residues, displayed similar reactivity to tau187, we wondered if protein length influences tau reactivity. We therefore tested the reactivity of a shorter tau construct. The 25-mer peptide (residues 310-335) was reacted in parallel with C291S and SKCGS reactions (Fig. 5). When combined with CA, this peptide (SKCGS with ten additional tau residues included on either side) was more reactive than SKCGS was and slightly less reactive than C291S was, suggesting construct length influences tau reactivity and that most of the excluded residues are not very involved in tau hyperreactivity.

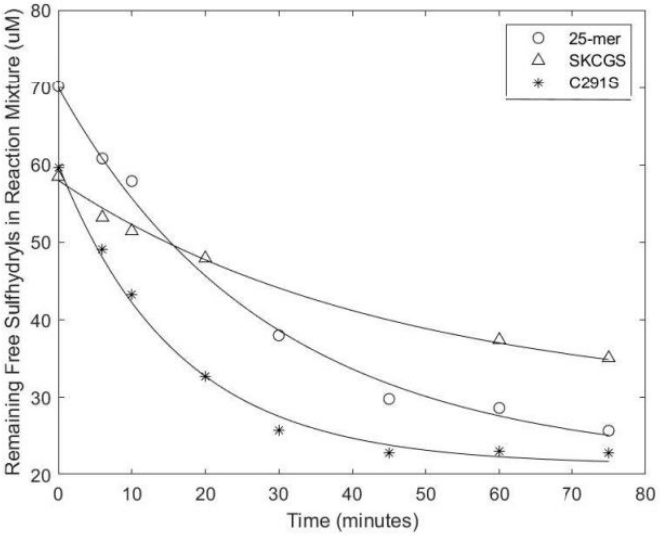

Figure 5. SKCGS vs C291S vs 25-mer at room temperature in 1X MOPS: 700 uM of CA was reacted with 70 uM of the 25-mer, 60 uM C291S, and 58 uM SKCGS and monitored over the course of 1.25 hours. Remaining sulfhydryl concentrations and curve fitting parameters were determined as described in the Materials and Methods section.

To find out if the increased reactivity around cysteines 291 and 322 was due to specific structural determinants near these positions or due to a general effect, we tested how well S413C reacts with CA in comparison to C291S. The S413C construct is a tau187 triple mutant with both cysteines 291 and 322 replaced by serines and the serine at 413 (no neighboring lysines and a different local environment) replaced by cysteine. The S413C reaction, done alongside a C291S reaction, resulted in association and dissociation constants (Table 1) that indicate S413C is about as reactive as C291S.

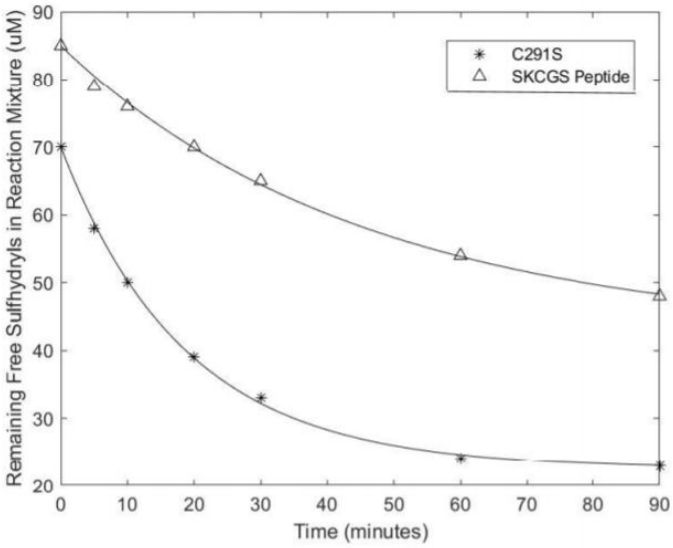

Figure 6. C291S vs SKCGS in Intracellular MOPS Buffer at room temperature (22°C): 70 uM C291S and 85 uM SKCGS were each reacted with 700 uM CA and monitored over the course of 1.5 hours at 22°C in intracellular MOPS buffer. The Intracellular MOPS Buffer (pH = 7.2) was 15 mM MOPS, 10 mM NaCl, 150 mM KCl, 0.5 mM $MgCl_2$, and 100 uM EDTA. Remaining sulfhydryl concentrations and curve fitting parameters were determined as described in the Materials and Methods section.

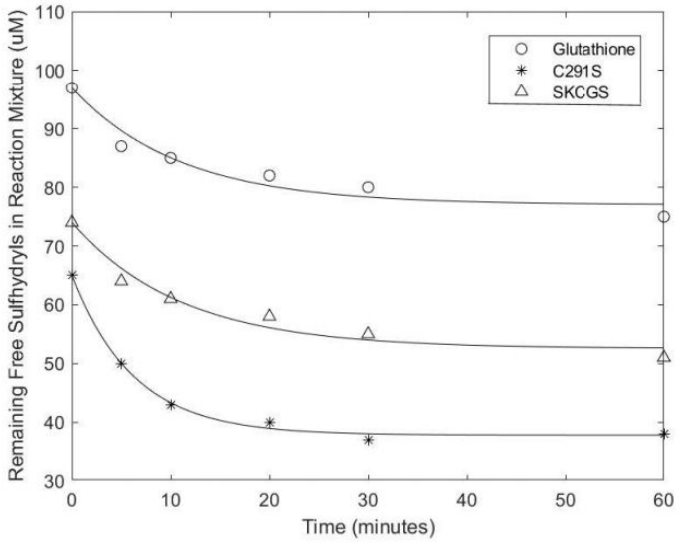

Figure 7. C291S, SKCGS, and GSH in Intracellular MOPS Buffer at physiological temperature (37°C): 65 uM C291S, 74 uM SKCGS, and 97 uM GSH were each reacted with 700 uM CA in a 37°C water bath and monitored over the course of an hour in intracellular MOPS buffer. Remaining sulfhydryl concentrations and curve fitting parameters were determined as described in the Materials and Methods section.

The effects of temperature and ionic strength on the reactivity of the sulfhydryl-bearing species were examined using C291S and the SKCGS peptide as representatives for tau-like and non-tau-like compounds, respectively.

Table 2. Intracellular MOPS Rate Constants

Temperature	Species	Replicates	Average Forward Rate Constant (e-05 uM^-1 min^-1)	Average Reverse Rate Constant (e-02 uM^-1 min^-1)	Equilibrium Dissociation Constant

Room temperature	C291S	15	6.87	2.47	360
	SKCGS	6	1.80	1.46	812
	GSH	3	0.75	0.43	574
Physiological Temperature	C291S	3	9.67	7.45	770
	SKCGS	3	2.90	3.97	1,370
	GSH	2	2.07	4.64	2,238

Intracellular MOPS buffer was meant to mimic a pH-controlled environment with an ionic strength that reflects the environment within a mammalian cell more accurately than MOPS buffer does. The ion concentrations that were used were obtained from *Cell Biology by the Numbers*[18]. Under these conditions, the CA reactions (Fig. 6) produced association and dissociation rates (Table 2) that were very similar to those of the experiments performed in MOPS buffer (Table 1).

Since physiological temperature is 37°C and previous CA-tau experiments were done on ice, we also wondered how changes in temperature would affect our observed kinetics. To find out, C29S, GSH, and SKCGS were first incubated in a 37°C water bath for 10 minutes then combined with CA, resulting in a reaction mixture with a pH of 6.88 (Fig. 7). Time points taken over a 1-hour timecourse revealed an increase in the association and dissociation rate constants for both species as well as increases in their respective equilibrium dissociation constants (Table 2).

Discussion

The results of this study point to an unusually high reactivity between tau and CA that seems to be specific to the tau protein molecule itself.

The difference in reactivities displayed by SKCGS when compared to tau is unexpected, because tau is an unfolded protein, in which secondary or tertiary structural determinants would not be expected to affect cysteine reactivity. The similarity between SKCGS and small molecule model compounds (Table 3) suggests tau is more reactive than the "average" sulfhydryl group, as opposed to SKCGS being less reactive than the average. SKCGS is in fact 2-fold more reactive than GSH and BME, and this can be accounted for by the lysine N-terminal adjacent to the cysteine, which our lab previously showed increases cysteine reactivity in tau[13]. This may be due to stabilization of the thiolate anion by the lysine positive charge[19].

Tau187 is approximately 20-21 kDa, and most cellular proteins including those of yeast are expected to be this molecular weight or larger. Yet, the average cysteine reactivity of yeast proteins is in fact similar to the much smaller SKCGS. The unique thiol reactivity of tau may therefore be critically influenced by its unfolded nature, which is its most unique characteristic compared to all other cellular proteins. It must be noted however that in a heterogeneous mixture of protein sulfhydryls, such as a yeast extract, our inability to differentiate the reactivities of each type of protein sulfhydryl present leaves open the possibility of there being present a hyperreactive protein thiol in small amount that the average reaction velocity would not be affected significantly.

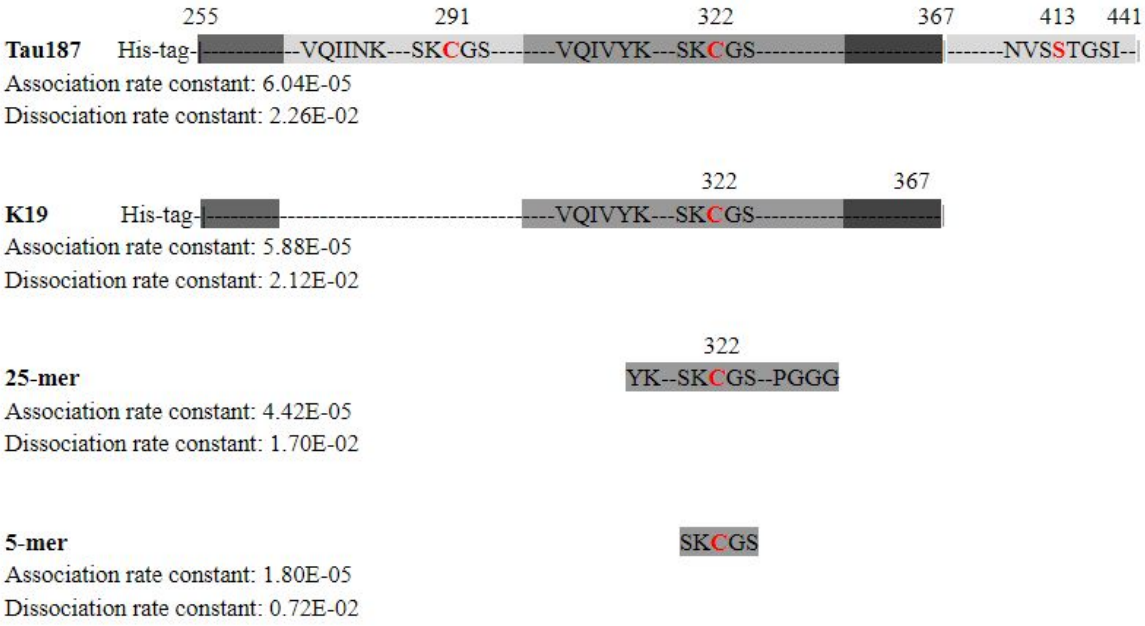

Figure 8. Construct illustrations and corresponding rate constants at 22°C in MOPS Buffer

Cysteine reactivity in tau seems to increase as the length of the polypeptide chain around the cysteine increases. The K19 construct lacks repeat 2 (Fig 8) and the tau187 C-terminus (Fig 8), and both K19 and C291S contain only cysteine 322. While we cannot currently say if the C-terminus or repeat 2 are determinant factors in tau hyperreactivity, the highly similar reactivities between K19 and C291S kinetics suggest that beyond a certain point, polypeptide length has little influence on tau cysteine reactivity. However, the slight decrease in reactivity of the 25-mer (12 tau residues on either side of cysteine 322), in comparison with C291S (Table 3), suggests the existence of a critical length of between 12 and 36 residues on either side of a tau cysteine over which changes in length are insignificant and under which reactivity is influenced.

The similarity in reactivity between tau187, C291S, and C322S suggests a lack of interaction between the two cysteines in tau since significant interaction between the two cysteines would change the overall rate constant of tau187.

The similarities between C291S and S413C reactivity suggest hyperreactivity is not confined to a specific region of tau since hyperreactivity was retained even when the examined cysteine was artificially placed in a different region of tau (Table 3). The slight decrease in reactivity between S413C and CA can be attributed to the lack of a neighboring lysine, since the presence of a basic residue nearby has been shown to promote sulfhydryl Michael addition[13], but otherwise reactivity between S413C and C291S is the same, which indicates that a cysteine in any part of the tau molecule should display hyperreactivity.

Table 3. A Comparison of Reactivity

Species	Association	Dissociation
Tau187	0.88	0.91
C322S	0.76	0.94
C291S	1.00	1.00
K19	0.86	0.86
S413C (C291S C322S)	0.68	0.71
25-mer	0.61	0.69
SKCGS Peptide	0.26	0.59
Yeast Extract	0.24	0.67
GSH	0.11	0.17
BME	0.07	0.11

The average association and dissociation rate constants for each species were normalized using the average association and dissociation constants for cysteine 322 (C291S) to obtain the tabulated ratios. Therefore, the closer a value is to one, the more similar the association or dissociation rate of a species is to that of cysteine 322

Although we are currently unsure of the cause tau hyperreactivity, it seems that the peptide length around the cysteine residue as well as tau's unfolded nature make a difference in reactivity. Additional factors that could contribute to thiol reactivity include

hydrogen-bond-donating residues nearby which could lower the pKa of cysteine and stabilize its thiolate form[19].

To obtain a rough picture of how tau's increased specificity over other sulfhydryls, including protein sulfhydryls, may result in a greater stoichiometry of tau modification by CA, we used the rate constant values obtained at 37°C in this study (Table 2) to perform simulations (Fig. 9). In such simulations, we included tau as well as the two major pools of thiol-bearing species found in a cell: protein sulfhydryls and GSH, the main intracellular reducing agent[20]. 2,500 uM of GSH thiol[21], 2,284 uM of non-tau protein thiol (estimated experimentally from a wild type mouse brain extract using Materials and Methods protocols) and 4 uM of tau thiol[22] were used as initial concentrations. The rate constants for SKCGS at 37°C were used to represent the kinetics of protein sulfhydryls; we did not test yeast extract at 37°C and since the yeast extract and 5-mer reacted similarly at room temperature, it was assumed that both would still behave similarly at 37°C.

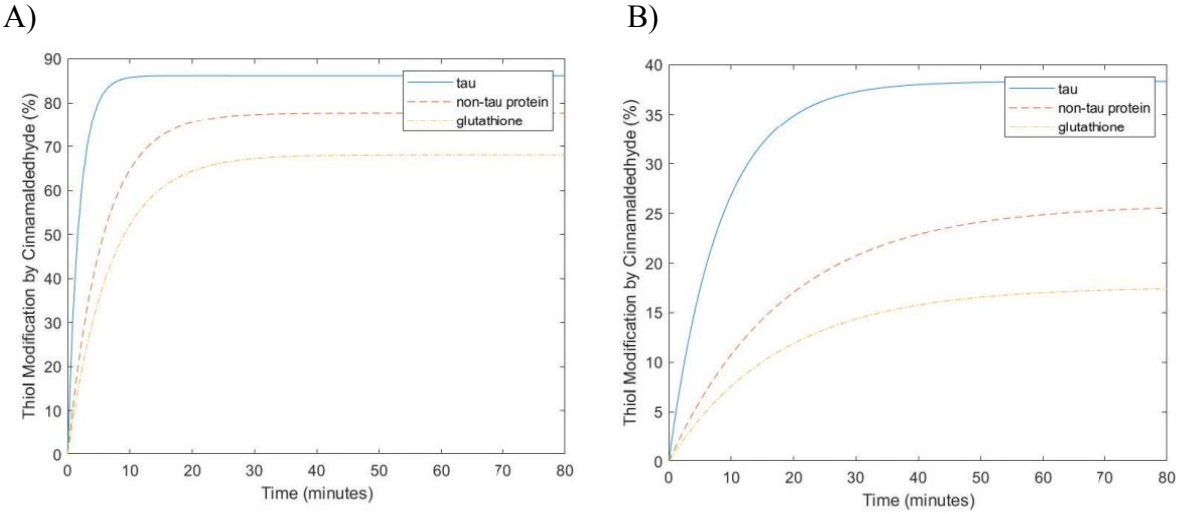

Figure 9. Simulated CA-Binding Competition Among Tau, Non-Tau Protein and GSH Thiols: A) Assuming a constant CA concentration of 4788 uM **B)** Assuming a constant CA concentration of 478.8 uM. Both simulations were done assuming the initial concentrations of 4 uM tau thiol, 2284 uM non-tau protein thiol, and 2500 uM GSH thiol. The association and dissociation rate constants of C291S, SKCGS, and GSH at 37°C and pH 6.9 were used to represent tau, non-tau protein, and GSH respectively. Simulations were done in Matlab

using a second order reversible model to simulate the number of thiols from each species that would be modified by CA over a course of 80 minutes. The concentration of CA-modified thiols at any point in time was graphed as a percentage of each initial free-thiol concentration.

Because so little is known about what happens to CA in the body, CA pharmacokinetics were not taken into account in our simulations. Instead, two sets of simulations were done, one with CA equimolar to the total thiol concentration (Fig. 9A) and another with CA ten-fold less than equimolar (Fig. 9B). However, both these concentrations (corresponding to 0.07 g/kg and 0.007 g/kg respectively for a 70 kg person) are still less than the estimated lethal dose of CA for humans (0.5-5 g/kg for a 70 kg person[23]). The CA concentration for both simulations was also kept constant, with the assumption that blood circulation would keep CA at a steady state. At both CA concentrations, a greater percentage of tau thiols was modified compared to those of non-tau proteins and GSH. With a constant concentration of 4788 uM CA, 86.1% of tau sulfhydryls were modified, whereas 77.8% of non-tau protein and 68.1% of glutathione thiols were modified. At a constant concentration of 478.8 uM CA, 38.3% of tau sulfhydryls were modified, whereas 25.5% of non-tau protein and 17.4% of glutathione thiols were modified. These percentages suggest CA has a preference for reacting with tau even in a mixture of proteins, and therefore as a drug in the body, CA may also be able to react preferentially with tau.

Overall, our results suggest that CA reaction with tau shows slight favorability over other sulfhydryl-bearing species including protein sulfhydryls and glutathione. CA already shows certain favorable characteristics as a possible small molecule inhibitor of tau aggregation *in vivo*. CA is fairly water-soluble, able to cross the blood-brain barrier, and non-toxic at moderate concentrations. In addition, the present work now shows that CA displays some specificity for reaction with tau in a complex mixture of protein sulfhydryls and glutathione,

such as the neuronal cytosol. With the added benefit of increased reaction specificity, CA may be a highly favorable candidate to guide new therapeutics that may inhibit pathological tau aggregation related to Alzheimer's disease.

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