JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Bioactivity Profiling with Parallel Mass Spectrometry Reveals an Assemblage of Green Tea Metabolites Affording Protection against Human Huntingtin and α-Synuclein Toxicity

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Aberrant protein aggregation and misfolding are key pathological features of many neurodegenerative disorders, including Huntington's and Parkinson's diseases. Compounds that offer protection from toxicity associated with aggregation-prone neurodegenerative proteins may have applications for the treatment of a multitude of disorders. A high-throughput bioassay system with parallel electrospray ionization mass spectrometry screening has been designed for critical evaluation of milligram quantities of natural product extracts, including dietary substances, for compounds of pharmacological relevance to the treatment of human neurodegenerative diseases. Using *Saccharomyces cerevisiae* strains engineered to express mutant human huntingtin and α-synuclein, we are able to identify extracts and compounds that protect cells from toxicity associated with these proteins. Applying this screening paradigm, we determined that a bioactive green tea extract contains an assemblage of catechins that were individually characterized for their respective protective effects against huntingtin and α-synuclein toxicity.

KEYWORDS: Huntington's disease; Parkinson's disease; huntingtin; α-synuclein; protein aggregation; natural products; protection; neuroprotective, green tea; catechins; bioassay; mass spectrometry; yeast; *Saccharomyces cerevisiae*

INTRODUCTION

Aberrant protein conformations lie at the heart of many human diseases impacting the central nervous system (*1*). Important examples of proteins that misfold and are associated with neurological disorders include α-synuclein (Parkinson's disease), huntingtin (Huntington's disease), amyloid-β (Alzheimer's disease), and PrP (prion-based disorders) (*2*). These and other neurotoxic proteins are highly prone to aggregation, forming remarkably similar fibril amyloids comprised of repeating cross β-sheet motifs (*1, 3*). It is hypothesized that aggregate-prone proteins exert their deadly effects through gain-of-toxic-function mechanisms in which several cellular processes are compromised (*3*).

Our laboratory has initiated studies to identify natural product-derived small molecules, including dietary phytochemicals, which offer protection against neurotoxic proteins. To date, we have focused our efforts on two families of protein-based neurodegenerative diseases: (1) CAG triplet repeat disorders typified by Huntington's disease (HD) and (2) synucleinopathies most notably characterized by Parkinson's disease (PD). Approximately 1 in 10000 people is diagnosed with HD which is caused by mutations occurring in the polyglutamine encoding region of *huntingtin* (*1, 4*). The CAG triplet repeat region of *huntingtin* normally encodes ~5–35 glutamine residues near the N-terminus of the protein. Huntingtin becomes highly prone to aggregation upon further expansion of the polyglutamine repeat region contributing to the development of HD symptoms, including chorea, dementia, and death. The age of onset and severity of HD are closely correlated with the degree of CAG triplet repeat expansion.

The frequency of PD is much greater, affecting as many as 1 in 100 people older than 65 years of age (*5*). A key feature of the disease is the formation of Lewy bodies containing α-synuclein (*6*). Molecular, animal model, and clinical evidence supports the assertion that α-synuclein misfolding has a

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10.1021/jf072241x CCC: $37.00 © 2007 American Chemical Society
Published on Web 10/19/2007

substantial contributory role in PD, and three familial α-synuclein mutants have been linked to early onset PD (*7–9*). However, the specific contributory role of α-synuclein and its mutant isoforms to cellular lethality has not yet been definitively delineated.

The yeast *Saccharomyces cerevisiae* has been developed as an exceptional experimental tool for high-throughput assay screening. This organism has been adopted as a robust cellular model system for studying human neurodegenerative disorders (*10, 11*), including HD (*12*) and PD (*13*). We have utilized transgenic *S. cerevisiae* strains expressing mutant human huntingtin and α-synuclein to systematically examine natural product sources for compounds offering protection against these proteotoxins. Accumulation of mutant human huntingtin and α-synuclein in *S. cerevisiae* results in the rapid onset of cell death, while treatment with bioactive natural products can provide cellular protection as demonstrated by increased growth. By combining HPLC fractionation in a microplate format with transgenic yeast-based bioassay screening and parallel electrospray mass spectrometry, we are able to rapidly assess the efficacy of natural product extracts and systematically target their respective biologically active constituents. Here we demonstrate how this integrated process has been applied to the initial assessment of the bioactive constituents present in a small (4 mg) catechin-enriched *Camellia sinensis* (L.) Kuntze (green tea) extract. Consumption of green tea has garnered considerable interest due to its reputed neuroprotective effects (*14*); however, only a limited number of selected green tea metabolites have been individually examined for their pharmacological effects. Our systematic, bioactivity-driven methodology has revealed (1) multiple green tea metabolites contribute to the protective properties of *C. sinensis* and (2) this comprehensive screening approach is a powerful tool for the rapid assessment of extracts and discovery of new neuroprotective agents from natural product sources.

MATERIALS AND METHODS

General Experimental Procedures. Loose green tea was obtained from R. C. Bigelow, Inc. (Fairfield, CT). Semipreparative HPLC was performed on a Shimadzu system, using a SCL-10A VP system controller, an SPD-10AV VP UV–vis detector, LC-10AT VP pumps, a DGU-14A solvent degasser, and an FRC-10A programmable fraction collector. Preparative HPLC was performed on a similar system using LC-6AD pumps. Phenomenex C_{18} Gemini semipreparative (5 μm, 110 Å, 250 mm × 10 mm) and preparative (10 μm, 110 Å, 250 mm × 21.2 mm) columns were used for HPLC. Electrospray ionization data were acquired on a LCT Premier (Waters Corp.) time-of-flight mass spectrometer. The instrument was operated at 10000 resolution (W mode) with dynamic range enhancement that attenuates large intensity signals. The cone voltage was 60 eV. Spectra were acquired at a pusher frequency of 16666 Hz covering the mass range of 100–1200 units and accumulating data for 2 s per cycle. Mass correction for exact mass determinations was done automatically with the lock mass feature in the MassLynx data system. A reference compound in an auxiliary sprayer is sampled every third cycle by toggling a "shutter" between the analysis and reference needles. The reference mass is used for a linear mass correction of the analytical cycles. Samples for MS were dissolved in MeOH and H_2O (9:1) with 0.2 mM ammonium formate (negative ion mode) or 0.5% by volume formic acid (positive ion mode). Samples were introduced via 20 μL loop injection using an auto injector (LC PAL, CTC Analytics AG). NMR data were obtained on a Varian VNMR system at 500 MHz (^{1}H) and 125 MHz (^{13}C). Optical rotations for all compounds were determined on an AutoPol III polarimeter. The optical densities (OD) of cultures in 96-well plates were obtained using a Tecan Infinite M200 microplate reader. Chemical standards for retention time analysis were purchased from Sigma-Aldrich, Inc., and TCI America.

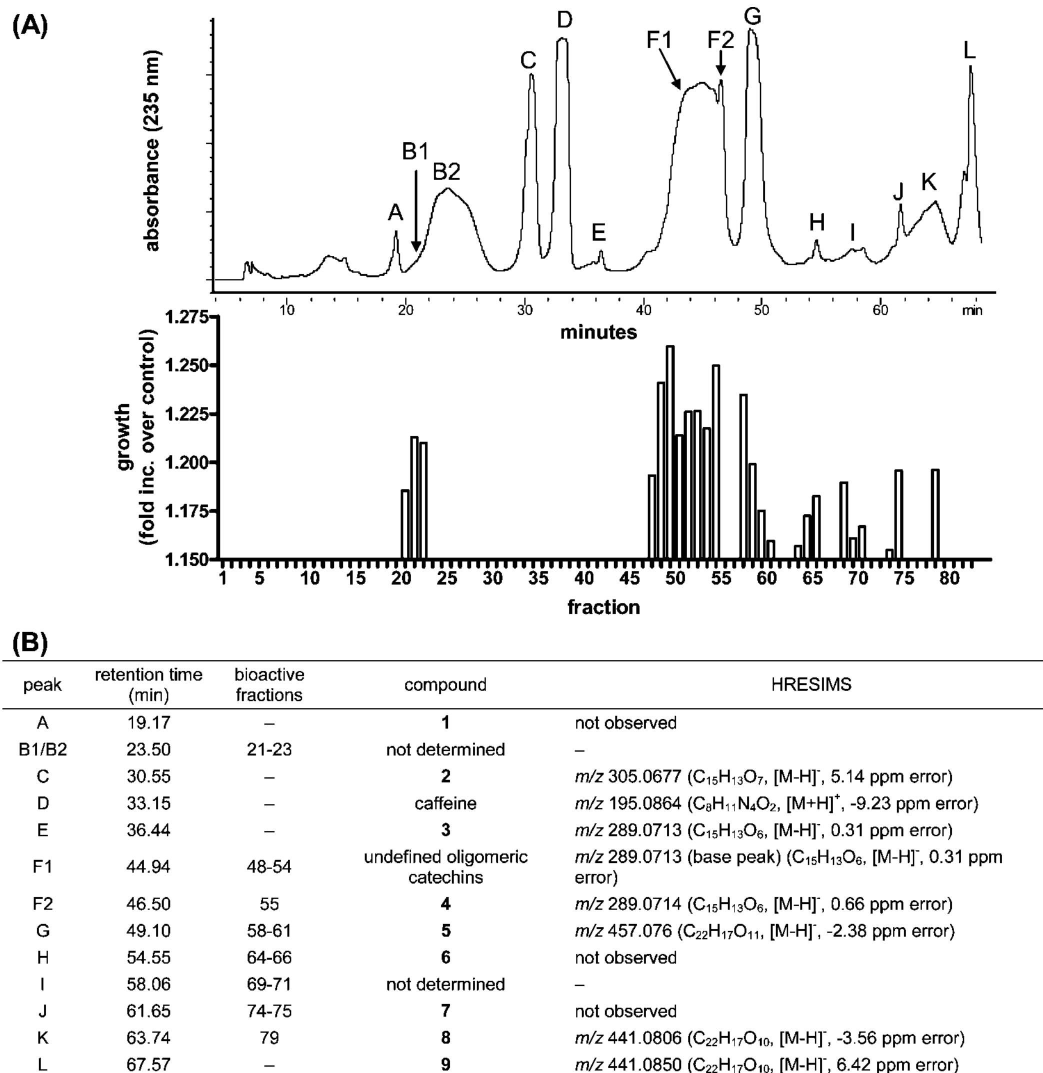
Yeast Strain and Plasmids. *S. cerevisiae* strain BY4741 (*MAT*α *his3*Δ *leu2*Δ *met15*Δ *ura3*Δ Δ*erg6*) was used for all assay strains. The *erg6* mutant strain BY4741 was chosen since the loss of this enzyme results in disruption of ergosterol biosynthesis, rendering the yeast plasma membrane more permeable to xenobiotics (*15*). Plasmids containing constructs for huntingtin expression were previously prepared (*12*) containing the 17 N-terminal residues of *huntingtin* exon 1 with a 25- or 103-polyglutamine repeat region (gift from M. Y. Sherman, Department of Biochemistry, Boston Medical School, Boston, MA). Huntingtin constructs were fused to a fluorescent enhanced green fluorescent protein (EGFP) tag under the control of a GAL1 promoter. The presence of the huntingtin expression products could clearly be observed by fluorescence microscopy (25Q-diffuse and 103Q-aggregated) and verified by PCR. Likewise, wild-type α-synuclein constructs (*16*) (gift from D. L. Gross, Health Science Center, Louisiana State University, Baton Rouge, LA) were linked with glutathione *S*-transferase (GST) for biochemical detection and the constructs placed under a GAL1 promoter before transformation into BY4741. Plasmids containing the huntingtin or α-synuclein constructs were transformed into *Escherichia coli* DH5α cells by heat-shock treatment (*17*) for amplification. Plasmids were obtained from overnight *E. coli* cultures using a Qiagen QIAprep Spin Miniprep Kit and transformed into *S. cerevisiae* cells using the lithium acetate heat-shock method (*18*). All cultures were maintained using standard microbiological techniques.

Huntingtin and α-Synuclein Assays. Yeast cultures were grown for 24 h in synthetic complete (SC) glucose medium without uracil. For the assay, yeast strains were pelleted by centrifugation and resuspended in SC galactose medium without uracil at the appropriate cell density (OD = 0.045 for the huntingtin strain and OD = 0.9 for the α-synuclein strain at 600 nm). The diluted cells and test samples dissolved in DMSO were added to the wells of sterile, polystyrene 96-well plates. The final DMSO concentration remained constant across all test and control wells and did not exceed 1% (v:v). An initial OD reading was immediately obtained ($t_{0\ h}$) at a wavelength of 600 nm, and the plates were placed in a shaker/incubator at 27 °C for 24 h. After 24 h ($t_{24\ h}$), the plates were removed from the incubator and the well contents gently mixed to ensure an even distribution of cells before the final OD was determined. The mean OD difference of the control wells from $t_{0\ h}$ to $t_{24\ h}$ was calculated for each strain ($\text{control}_{t_{24\ h}} - \text{control}_{t_{0\ h}}$). These values were subtracted from the average change in OD for each test sample concentration ($\text{test}_{t_{24\ h}} - \text{test}_{t_{0\ h}}$) in the determination of the overall percent change in OD for each treatment. This assay was developed with adherence to the recommendations as outlined in the NIH Assay Guidance Manual (*19*). Appropriate uniformity tests for interplate and interday variation, signal to noise, and efficacy were performed on untransformed OD data and have proven to be acceptable.

The mammalian (PC12) cell-based assay was performed using an N-terminal huntingtin fragment with an expanded polyglutamine region. Details of this assay have been described previously (*20*).

Microplate Bioassay and ESI-MS Screening. A 4 mg sample of the green tea ethyl acetate fraction was subjected to semipreparative HPLC and the eluted material collected as 1.5 mL fractions in 84 wells of a deep well 96-well microplate. Half of the eluant from each fraction was transfered to a second microplate and the solvent removed from both plates. The dried fractions in one plate were dissolved in DMSO and 2 μL aliquots transferred to an assay plate for biological testing. The contents of the second plate were analyzed via analytical ESI-MS in both positive and negative ion modes. The resultant data were then combined to provide an assessment of the fractions affording protection from huntingtin and α-synuclein toxicity.

Scale-Up Extraction and Isolation of Green Tea Metabolites. Dried and ground green tea leaves (19 g) were extracted twice over a 32 h period with MeOH and dried in vacuo. The crude extract (5.8 g) was initially redissolved in aqueous MeOH and partitioned against hexane, CH_2Cl_2, and EtOAc in a modified Kupchan-like extraction. The EtOAc fraction (1.61 g) was found to contain the bioactive green tea metabolites and was further fractionated. Preparative and semipreparative gradient HPLC with acetonitrile and H_2O resulted in the isolation of gallocatechin (**1**) (4 mg), epigallocatechin (**2**) (114 mg), catechin (**3**) (5 mg), epicatechin (**4**) (37 mg), epigallocatechin 3-*O*-gallate (**5**) (199 mg), epigallocatechin 3-*O*-(3′-*O*-methyl)gallate (**7**) (7



peak	retention time (min)	bioactive fractions	compound	HRESIMS
A	19.17	–	**1**	not observed
B1/B2	23.50	21-23	not determined	–
C	30.55	–	**2**	*m/z* 305.0677 ($C_{15}H_{13}O_7$, $[M-H]^-$, 5.14 ppm error)
D	33.15	–	caffeine	*m/z* 195.0864 ($C_8H_{11}N_4O_2$, $[M+H]^+$, -9.23 ppm error)
E	36.44	–	**3**	*m/z* 289.0713 ($C_{15}H_{13}O_6$, $[M-H]^-$, 0.31 ppm error)
F1	44.94	48-54	undefined oligomeric catechins	*m/z* 289.0713 (base peak) ($C_{15}H_{13}O_6$, $[M-H]^-$, 0.31 ppm error)
F2	46.50	55	**4**	*m/z* 289.0714 ($C_{15}H_{13}O_6$, $[M-H]^-$, 0.66 ppm error)
G	49.10	58-61	**5**	*m/z* 457.076 ($C_{22}H_{17}O_{11}$, $[M-H]^-$, -2.38 ppm error)
H	54.55	64-66	**6**	not observed
I	58.06	69-71	not determined	–
J	61.65	74-75	**7**	not observed
K	63.74	79	**8**	*m/z* 441.0806 ($C_{22}H_{17}O_{10}$, $[M-H]^-$, -3.56 ppm error)
L	67.57	–	**9**	*m/z* 441.0850 ($C_{22}H_{17}O_{10}$, $[M-H]^-$, 6.42 ppm error)

Figure 1. Bioactivity profiling results for a green tea extract in the huntingtin assay. (**A**) A 4 mg sample of green tea was subjected to semipreparative HPLC with acetonitrile and H_2O (10–25% acetonitrile, 70 min), generating 84 fractions that were evaluated for their protective effects against toxicity associated with mutant huntingtin expression. (**B**) Accurate mass ESI-MS analysis of the microplate fractions was used to tentatively identify the bioactive compounds which were subsequently confirmed through spectroscopic comparison to the scale-up isolates and commercial standards.

mg), and epicatechin 3-*O*-gallate (**9**) (58 mg). All compounds isolated by scale-up procedures were identified by accurate mass ESI-MS, ^{1}H NMR, comparison of HPLC retention times versus those of commercial standards, and optical rotation data. All data matched those published previously (*21*), and ^{1}H NMR spectra are provided as Supporting Information.

RESULTS

Bioassay screening of our in-house natural product extract library resulted in the identification of a green tea sample that conveyed protection against toxicity associated with mutant human huntingtin and α-synuclein expression in a yeast-based assay system. Applying our semiautomated bioassay profiling approach (**Figure 1**), we subjected 4 mg of the EtOAc fraction to C_{18} HPLC, generating 84 samples that were deposited as 1.5 mL aliquots into a 96-well plate. The fractions were dried and dissolved in DMSO prior to being delivered to the daughter test plates.

Bioassay profiling results implicated several fractionated compounds in providing protection against the transgenic huntingtin and α-synuclein toxicity. Data generated from the huntingtin assay are shown in **Figure 1A**. From these data, several distinct regions of the resultant chromatogram were clearly linked to bioactive wells in the 96-well test plate. Analysis of the initial ESI accurate mass data corresponding to the wells of the fractionated sample supported the assertion that catechin derivatives (**Figure 2**) were the bioactive species (**Figure 1B**). Bioactive fractions F1, F2, G, and K were tentatively identified as containing a mixture of undefined oligomeric catechins (*m/z* 289.0713, base peak, $C_{15}H_{13}O_6$, $[M - H]^-$ plus other polymeric species), **4** (*m/z* 289.0714, $C_{15}H_{13}O_6$, $[M - H]^-$), **5** (*m/z* 457.076, $C_{22}H_{17}O_{11}$, $[M - H]^-$), and **8** (*m/z* 441.0806, $C_{22}H_{17}O_{10}$, $[M - H]^-$), respectively. Mass spectral screening of the remaining wells indicated the presence of related catechin species, including **2** (*m/z* 305.0677, $C_{15}H_{13}O_7$, $[M - H]^-$), **3** (*m/z* 289.0713, $C_{15}H_{13}O_6$, $[M - H]^-$), and **9** (*m/z* 441.0850, $C_{22}H_{17}O_{10}$, $[M - H]^-$).

To confirm the assignments proposed above and to obtain sufficient material for pharmacological testing, we performed scale-up isolation using preparative and semipreparative gradient HPLC on 1.61 g of the EtOAc fraction derived from 19 g of plant material. Information gleaned from the chromatographic trace and ESI-MS data in **Figure 1** facilitated the rapid procurement of seven green tea compounds (**Figure 2**) that exhibited varying degrees of protection against human huntingtin

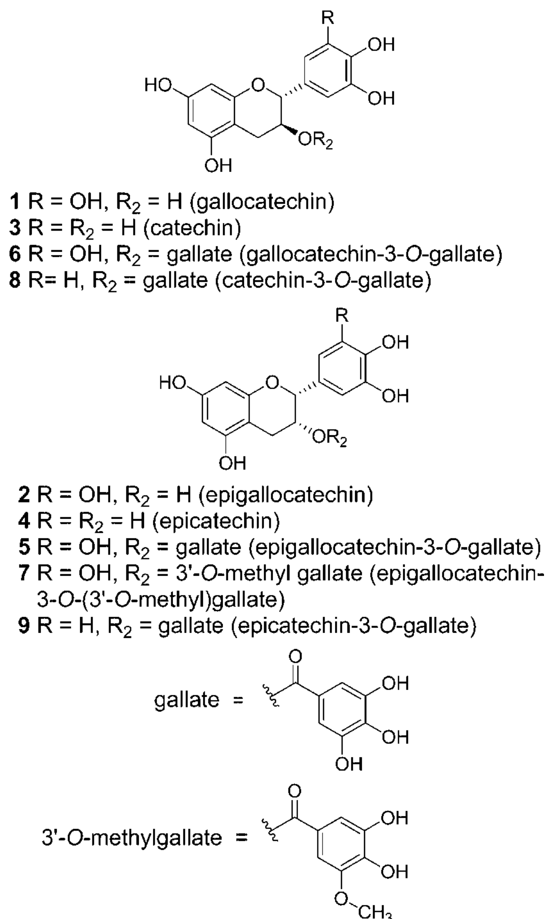


Figure 2. Structures of green tea metabolites discussed in this study.

and α-synuclein toxicity. All of the catechins were readily identified by means of dereplication using MS, ^{1}H NMR (*21*), comparison of HPLC retention times versus those of commercial standards, and optical rotation data (^{1}H NMR spectral data of the scale-up isolates are available as Supporting Information). In the course of this work, caffeine was also isolated and confirmed as being inactive.

The protective effects of the purified green tea metabolites against human huntingtin and α-synuclein expression are shown in **Figures 3** and **4** respectively. Despite similarities in the cellular toxicities associated with the two neurodegenerative proteins, the green tea isolates exhibited different bioactivity profiles against huntingtin and α-synuclein. Whereas compounds **1** and **2** exhibited substantial protection against mutant huntingtin-induced toxicity, neither compound was active in the α-synuclein model system. In contrast, similar activity profiles were noted for some of the metabolites in both model systems. It is interesting that compound **3** was completely inactive against both proteotoxins while its epimer, **4**, was active, providing substantial protection from mutant huntingtin and α-synuclein expression. The addition of a gallate moiety to compound **4**, as present in isolate **9**, had little effect on protection against mutant huntingtin toxicity, but that addition showed a reduced potency with respect to α-synuclein. Remarkably, the addition of a gallate moiety to compound **2** to yield metabolite **5** did not change the potency against mutant huntingtin, whereas it transformed this catechin to a more potent protective agent against α-synuclein. It is noteworthy that isolate **7**, a methylated gallate derivative of compound **5**, exhibited the greatest potency against α-synuclein and was also highly active against huntingtin toxicity. This appears to be the first report of any potential antineurodegenerative or neuroprotective activity attributable to a catechin containing an *O*-methyl gallate moiety.

As a result of these findings, we were interested in determining whether the catechins may have promising activity in a mammalian system. Previous reports had suggested that catechins and their metabolites are capable of entering the brain (*22, 23*); however, the amounts detected were rather minute. Accordingly, we chose to limit our testing of the catechins in the mammalian cell model for HD to a concentration range that would be more in accordance with what could be expected from oral administration of the green tea metabolites. At a maximal dose of 10 μM, none of the catechins exhibited protection against toxicity associated with mutant huntingtin expression. Further experiments aimed at identifying more potent analogues that are active in the mammalian cell model through derivatization of the available isolates, synthesis of bioactive catechin mimetics, and procurement of additional minor secondary metabolites from other green tea fractions are underway.

DISCUSSION

The *S. cerevisiae* models of huntingtin and α-synuclein expression are effective tools for the screening and detection of potential neuroprotective natural products that may be used for the pharmacological treatment of HD and PD. The advantages of the yeast-based bioassay-driven methodology used in this study are several-fold. The foremost of these is that we can readily screen for related compounds on the basis of bioactivity (bioassay) and chemical structure (mass spectrometry) in parallel. This approach is highly sensitive, requiring only minute quantities of material so that crude extracts can be examined quickly and in a cost-effective manner. Furthermore, the yeast models are ideal in light of their ability to recapitulate many physical and biochemical aspects of protein aggregation-related disease states (*10*). All of these features make the yeast huntingtin and α-synuclein expression systems exceptional tools for the discovery of new protective agents for combating human neurodegenerative disorders such as HD and PD. We are continuing to further refine our high-throughput microplate screening methodology so that we can more accurately detect bioactive compounds at even lower concentrations, and we are improving our mass spectral identification of metabolites in individual microplate wells.

A very intriguing relationship has emerged in recent years regarding the potential impact of green tea metabolites upon neurodegenerative disease treatment and prevention. Compound **5** [epigallocatechin 3-*O*-gallate (EGCG)] has garnered a considerable portion of this evolving interest due in part to its multifaceted biological effects and quantitative prevalence among the green tea catechins. In a cell-free PrP conversion assay, compound **5** and other catechins performed exceptionally well, exhibiting IC_{50} values in the low (<10 μM) micromolar range (*24*). In vitro tests examining the effect of **5** against amyloid-β and α-synuclein aggregation demonstrated its ability to effectively inhibit filament formation with IC_{50} values of 2 and 9.8 μM, respectively (*25*). As an extension of these findings, it is noteworthy that related catechins **3** and **4** not only inhibited formation of fibrils of amyloid-β and α-synuclein but also have the ability to destabilize preformed fibrils (*26*). These findings are particularly important since the smaller oligomeric forms of these conformationally altered proteins, not the larger fibrillar aggregates, may represent the toxic species in vivo (*1, 27, 28*).

Protective effects of compound **5** have been noted in cell-based and whole animal assay systems. A yeast-based α-sy-

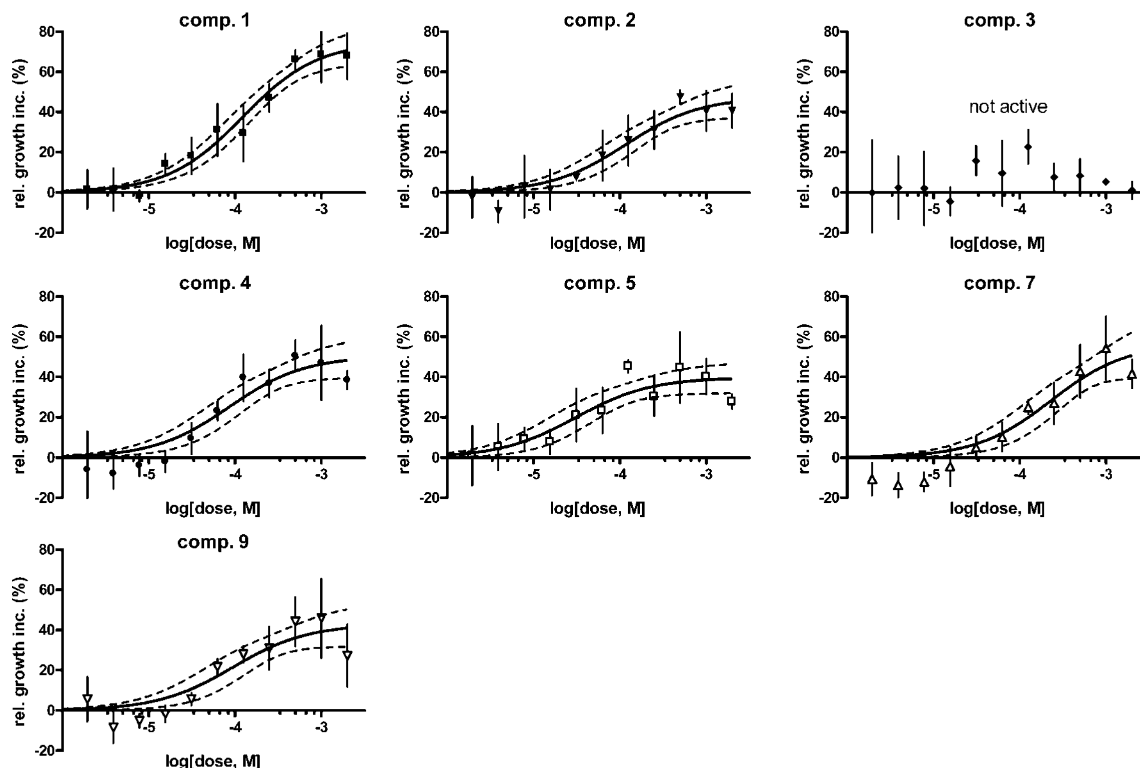


Figure 3. Dose–response protective effects of purified green tea isolates **1–5**, **7**, and **9** against toxicity associated with huntingtin expression. Data represent the means ± the standard deviation of three replicate doses. A sigmoidal dose curve of best fit (solid line) is shown along with their respective 95% confidence intervals (dashed line) for each compound. Data were likewise confirmed in independent experiments performed on at least one additional day.

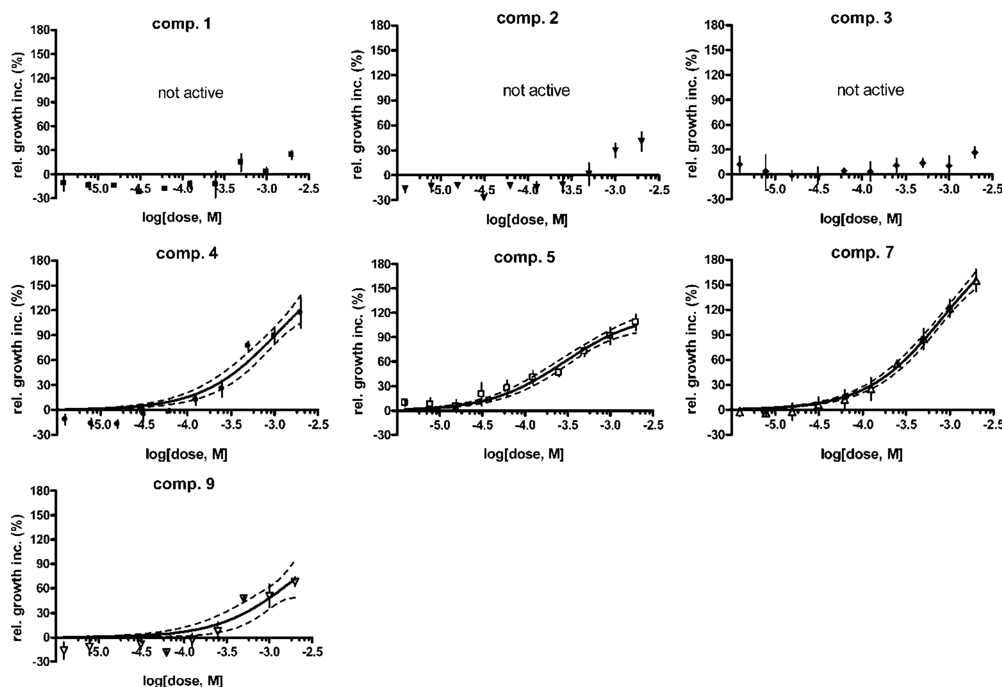


Figure 4. Dose–response protective effects of purified green tea isolates **1–5**, **7**, and **9** against toxicity associated with α-synuclein expression. Data represent the means ± the standard deviation of three replicate doses. A sigmoidal dose curve of best fit (solid line) is shown along with their respective 95% confidence intervals (dashed line) for each compound. Data were likewise confirmed in independent experiments performed on at least one additional day.

nuclein expression system was previously noted to be protected from toxicity following treatment with **5** (*29*). Likewise, huntingtin toxicity in yeast and fruit flies was reduced by administration of catechin **5** (*30*). Additionally, rat hippocampal cells were protected from amyloid-β toxicity as a result of administration of compound **5** (*31*). While the in vitro studies (vide supra) point to direct modulatory effects of **5** on protein aggregation as a means of protecting cells, several indirect mechanisms, including metal chelation (*32*), blockade of NO-induced cell apoptosis (*33*), and upregulation of oxidative stress response factors (*34*), along with others (*35*), for imparting the protective effects of **5** to cells and in vivo have been proposed.

Curiously, despite the diverse array of catechins and related phenolic species found in tea, only compound **5** has been subjected to significant scientific scrutiny to the exclusion of many other promising bioactive secondary metabolites. This has had some unfortunate consequences since studies performed using a limited numbers of catechins (*24, 29–31*) have led to potentially erroneous conclusions regarding the structure–activity relationships of this metabolite family. As seen in this study and intimated by others (*25*), the catechins possess a number of unique structural features requiring further medicinal chemistry studies for the full elucidation of the pharmacophore responsible for their protective properties. Our results strongly suggest the potential protective benefits of the catechins must be considered in light of the total assemblage of biomolecules present and that even more potent analogues (as observed in several fractions from the 96-well plate analysis) may have biologically significant effects. We are hopeful these studies will lead to a rational utilization of the catechins as protective agents against neurodegenerative diseases like HD and PD and as molecular tools for unraveling the pathogenesis of mutant huntingtin- and α-synuclein-associated toxicity.

ACKNOWLEDGMENT

We are grateful for the gifts of plasmids from M. Y. Sherman and D. L. Gross. We thank A. West for assistance and advice regarding the transformation of the yeast strains. Assistance from K. Langer and J. McCraw in obtaining the green tea samples from R. C. Bigelow, Inc., is greatly appreciated.

Supporting Information Available: Spectral data for the green tea isolates. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review July 25, 2007. Revised manuscript received September 13, 2007. Accepted September 14, 2007. Support from The University of Oklahoma College of Arts and Sciences and the Department of Chemistry and Biochemistry faculty start-up funds is greatly appreciated (R.H.C.). This project was also supported by funding from the NIH (NINDS Grant 16375) (C.A.R.). The MS instrumentation was obtained with support from NIH Grant SIG S10 RR019398 (T.D.W.).

JF072241X