Neuroprotective Effect of Brazilin on Amyloid β (25−35)-Induced Pathology in a Human Neuroblastoma Model

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ABSTRACT: Until the recent past, the sole exemplar of proteins as infectious agents leading to neurodegenerative disorders remained the prion protein. Since then, the self-seeding mechanism characteristic of the prion protein has also been attributed to other neurodegenerative-disease-associated proteins, including amyloid-β (Aβ), tau, and α-synuclein (α-Syn). In model cell line studies, truncated Aβ, viz. amyloid beta (25−35), has been found to influence cellular homeostasis through its interactions with, and via, the disruption of key housekeeping machinery. Here, we demonstrate that the incubation of human neuroblastoma (SH-SY5Y) cell line with Brazilin ((6aS,11bR)-7,11b-dihydro-6H-indeno[2,1-c]chromene-3,6a,9,10-tetrol) prior to Aβ (25−35)-insult protected the cells from oxidative stress and apoptotic cell death. Furthermore, Brazilin mitigated Aβ-induced alterations in protein disulfide isomerase (PDI) and α-synuclein status, both of which are important biomarkers that report on Parkinson’s pathogenesis. The results obtained in this study suggest that the tetrol is neuroprotective and helps resist Aβ-induced cross-pathology and amyloidogenic onset.

INTRODUCTION

Over the past few years, convincing evidence has demonstrated that several amyloid-dependent disorders can be transmitted by a prion-like mechanism.1,2 Cellular and animal models of diverse neurodegenerative disorders have implicated α-synuclein, amyloid-β (Aβ), tau, and polyQ mutant Huntingtin (mHTT) among others as being able to seed their associated cellular housekeeping machinery, and increased ubiquitination of misfolded debris.3−5 The results from the model cell line studies suggest that Aβ (25−35) contributes to cross-toxic outcomes via (at least) two different pathways.21

As stated before, what is less clear is how much this seed-spread mechanism eventually contributes to, and drives, “heterotoxicity”, a process by which a pathogenic seed infiltrates a non-native cellular milieu, corrupts cellular processes therein, and initiates a seemingly unrelated neurodegenerative cascade.22 However, this notion is not new. There already exists evidence that appears to indicate potential in these self-templating vectors to “cross-fertilize” unrelated neuropathies, at times, in neuronal regions distinct from their point of origin. For example, in AD, Aβ aggregates have been found to be copathological with TDP-43 cytoplasmic inclusions (over 50% of the cases) and with α-Syn Lewy neurites and LBs (over 40% of cases).23 Conversely, α-Syn species (RNS). Furthermore, Aβ (25−35) insult results in the chemical modification of PDI catalytic thiols, upregulation of cellular housekeeping machinery, and increased ubiquitination of misfolded debris.3−5 The results from the model cell line studies suggest that Aβ (25−35) contributes to cross-toxic outcomes via (at least) two different pathways.21

Received: March 2, 2020
Accepted: May 18, 2020
Published: June 4, 2020
pathology in Lewy bodies has been found to co-occur with Aβ pathology (>80% of the cases), with NFT and NT at Braak stage > II (over 50% of cases) and with TDP-43 pathology (over 30% overlap).24–28

Brazilin ((6αS,11bR)-7,11b-dihydro-6H-indeno[2,1-c]-chromene-3,6a,9,10-tetrol) from Caesalpinia sappan is an established antioxidant.29,30 Brazilin, as a phenolic antioxidant, has a potent inhibitory effect against Aβ (25−35) neurotoxicity. Here, we examine its role in preventing amyloid-β (Aβ)-induced toxicity, aggregation, and in modulating the Aβ-dependent aggregation pathway of other amyloid proteins.

## RESULTS AND DISCUSSION

### Dynamic Light Scattering (DLS)

Figure 1 depicts the size of Aβ (25−35) in solution measured using dynamic light scattering. In accord with previous studies, Aβ (25−35) was found to be (1.0–1.5 nm) below concentrations of 100 μM, beyond which it was found to form aggregates (Figure 1).31,32

**Figure 1.** Size of the Aβ oligomer preparation. This graph depicts the presence of the oligomeric size distribution intensity with a diameter of ~1.0−1.5 nm (the two small peaks to the left), whereas the third peak of the graph (to the right) corresponds to the formation of protofibrils.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Assay. For the specific antioxidant activity of Brazilin, we tested the in vitro radical scavenging ability of Brazilin measured by the diminution in the UV absorbance maximum of the DPPH radical. Brazilin in DMSO at concentrations 2.5 and 5 μM was able to quench the DPPH radical absorbance, suggesting that, at both concentrations, the antioxidant was capable of reducing the reactive oxygen species stress (Figure 2). The percentage of DPPH radical inhibition was found to be 65.7% at 2.5 μM and 79.5% at 5 μM, using ascorbic acid as a reference.33

**Figure 2.** Brazilin radical scavenging activity. The graph shows free radical scavenging activity at 2.5 and 5.0 μM. Both concentrations were able to decrease the absorbance obtained from the free radical solution, 1,1-diphenyl-2-picrylhydrazyl (DPPH).

### Cytotoxicity of Brazilin, Aβ (25−35), and Cotreatment

Cytotoxicity of Brazilin, Aβ (25−35), and Cotreatment. The cytotoxicity profile of Brazilin (1% v/v DMSO) in the cell line was established by measuring the percentage of cell death as a function of Brazilin (Figure 3). Utilizing the unbiased dose–response graph, concentrations that were found not to be cytotoxic to the SH-SYSY cell line were used for further experiments. There was no difference between “untreated” vehicle control and Brazilin treatment on SH-SYSY cells up to a concentration of 5 μM Brazilin. Aβ (25−35) cytotoxicity was also independently evaluated at 24 h after the introduction into the cells. The results reveal a smooth, dose-dependent increase in cytotoxicity (Figure 4). Figure 4B–D demonstrates how cell morphology is altered as a function of Aβ (25−35). Clear changes in morphology are observed between 10 and 35 μM of the added peptide, 24 h after incubation (Figure 4C,D).34 Also, the results suggest that Brazilin is able to protect cells from the cytotoxic effects of Aβ (25−35) (Figure 5). The results indicate that the protective effects of Brazilin are observed only at a concentration of 5 μM Brazilin.
across the concentration range of Aβ (25–35) tested (0–5 μM).

Figure 5. Brazilin (Braz) rescues amyloid-β (25–35) toxicity. Cytotoxicity of Brazilin showing its protective effect against Aβ (25–35) insult at different concentrations after 24 h of treatment. Data are mean values ± SD; differences were established with Student’s t-test compared to those of the vehicle group.

Defining the Role of Brazilin in the Interaction of α-Synuclein and PDI Aggregation Induced by Aβ (25–35). Aβ (25–35) is known to disrupt cellular homeostasis and the solubility status of proteins. These include α-Syn, synphilin-1 (Synp-1), and PDI. We determined whether Brazilin was able to maintain homeostasis under Aβ (25–35) insult. The addition of 20 μM Aβ (25–35) resulted in an increased expression of the housekeeping oxidoreductase chaperone compared to that of the untreated and vehicle controls as a result of cellular stress (Figure 6). By contrast, results indicate that SH-SY5Y cells treated with Brazilin (2.5 and 5 μM) for 1 h followed by the addition of Aβ (25–35) and subsequent incubation for 24 h attenuated the expression of the peptide on PDI expression levels; i.e., the presence of Brazilin (2.5 and 5 μM) caused a reduction of aggregate expression levels when compared with that of the 20 μM Aβ (25–35) insult. Both concentrations of applied Brazilin had statistically significant impacts (Figure 6A).

As previously reported, Aβ (25–35) upregulated the levels of α-Syn in SH-SY5Y cells (Figure 6B). However, unlike with PDI, the presence of Brazilin does not attenuate the α-Syn level at the tested concentrations of the polyphenol.

Impact of Brazilin against Aβ (25–35)-Induced Apoptosis. Aβ (25–35) insult to SH-SY5Y precipitates apoptosis. We examined whether Brazilin at 2.5 and 5.0 μM conferred neuroprotection by mitigating cellular apoptosis and necrosis. The bars in Figure 7 represent untreated cells; cells treated with DMSO (1% v/v); cells treated with Brazilin at 2.5 μM; Brazilin at 5.0 μM; Brazilin at 5.0 μM + Aβ 50 μM; and H2O2 100 μM. Note that we observed batch-to-batch differences in the cytotoxic potential of Aβ. Hence, here we used Aβ (25–35) 50 μM instead of 20 μM used previously. However, the presence of appropriate controls serves to account for differences in concentrations used. Green bars represent the total percentage of apoptotic cells, which is expressed as the sum of the lower-right quadrant (early apoptosis) and the top-right quadrant (late apoptosis) (Figure 7A). Cells positive to PI and negative to Annexin V-FITC are represented as red bars, and they are defined as the necrotic cell subgroup.

The results indicate that 50 μM of Aβ (25–35) induces apoptosis (high statistical significance was found when the DMSO control, 50 μM of Aβ (25–35), and 2.5 μM Braz + Aβ (25–35) treatment conditions were compared). Brazilin at both 2.5 and 5.0 μM was able to rescue the cells from 50 μM of Aβ (25–35)-induced apoptotic cell death. Cells treated with 100 μM of H2O2 were used as the positive control, in which an increase in early, late apoptosis, and necrosis was demonstrated.

Fluorescence Inhibition of Lysozyme Fibrillation. We tested whether Brazilin can directly inhibit protein fibrillation. Lysozyme was used as a model fibril-forming protein. Fibre presence was assessed by Thioflavin T (ThT). In Figure 8, the fluorescence intensity of the control (lysozyme fibrils alone) is greater than when Brazilin was introduced at the start of the lysozyme fibril-forming process. The reduction in the fluorescence intensity suggests that Brazilin (5 μM) functions as a prophylactic and can mitigate the formation of amylloid-like fibrils in proteins.

CONCLUSIONS

The presence of distinct biomarkers in AD and PD have led to their historical qualification as two different neurodegenerative disorders. Yet, cross-toxicity has been reported, whereby one amyloid protein associated with a particular neuropathy can trigger a seemingly unrelated pathology. In this study, we demonstrate that cross-pathology induced by Aβ (25–35) can be mitigated by Brazilin, a natural polyphenolic compound extracted from C. sappan. Our results reveal that the polyphenol can reduce reactive oxygen species, protect cells from Aβ toxicity, maintain cellular homeostasis, and inhibit protein aggregation of amyloidogenic proteins. The standard DPPH assay confirmed Brazilin as a potent antioxidant that can scavenge free radicals. Using hen egg-white lysozyme, a model amyloid fibril-forming protein as an exemplar, ThT fluorescence assays established the inhibitory effect of Brazilin on its amyloid-forming trajectory. Additionally, cell viability assays reveal that Brazilin was able to maintain cell homeostasis when co-incubated with Aβ (25–35), which was found to increase the expression of oxidoreductase chaperone and enhance cellular stress. Brazilin not only protected cultured cells from formation of aggregates but also mitigated the cellular apoptotic and necrotic death. Finally, the approach investigated in this study will help develop a comprehensive understanding of the cross-toxic origins promoted by amyloidogenic proteins. This work is not only a stepping stone to furthering our understanding of the players involved in cross-pathologies but provides a platform with which to test prophylactics that may eventually advance/improve therapeutic outcomes.

METHODS

Chemicals, Cell Line, and Reagents. The following reagents were sourced commercially and used without further purification. Brazilin, propidium iodide (PI), and hexafluorosipropanol (HFIP) (MP Biomedicals 154862, 19548); human Aβ 25–35 (Ana Spec AS-24448); Annexin V kit containing Annexin V-FITC and PI (Beckman Coulter); 1,1-diphenyl-2-picryl-hydrazyl (DPPH; Millipore-Sigma USA); Hoechst 33342 (Life Technology H1399); trypsin-EDTA 0.25% (Life Technologies, 25200-056); protein disulfide isomerase (PDI) antibody, and α-synuclein (Cell Signaling Technology; C81H6, 2647). Most of the secondary antibodies were obtained from Abcam (Alexa Fluor 488, Texas red; 13787 https://dx.doi.org/10.1021/acsomega.0c00396 ACS Omega 2020, 5, 13785–13792
ab150077, ab6787), and Fluoro-Gel II Mounting Medium was obtained from Fisher Scientific and Thioflavin T (CAS 2390-54-7) were purchased from Sigma-Aldrich.

Cell Culture. Human neuroblastoma cells (SH-SY5Y) (ATCC, Manassas, VA) were grown in a culture medium (DMEM/F-12, 398225 SIGMA) and supplemented with 10% fetal bovine serum. Prophylactically, 1% v/v of penicillin/streptomycin (15-140-122; Gibco) was added to the medium. Cells were maintained by incubation at 37 °C with 5% carbon dioxide. All experiments were performed by seeding the cells into 96-, 24-, 12-, and 6-well plates. Once confluent, the cells were then pretreated with Brazilin (2.5 and 5.0 μM) for 1 h, followed by Aβ (25–35) addition (20, 50 μM) and incubated for 24 h.

Preparation of Aβ 25–35. Briefly, human Aβ (25–35) was dissolved in HFIP, aliquoted, and incubated at room temperature for at least 30 min. The HFIP was allowed to evaporate, and the aliquots were stored at −20 °C. Immediately prior to use, Aβ (25–35) was dissolved in DMSO. The size (monomer, oligomer, fibril) of Aβ (25–35) in solution was periodically measured using dynamic light scattering (DLS), as previously described.

Radical Scavenging Activity by DPPH Method. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed as previously described. Briefly, 2 mM of DPPH was freshly prepared in methanol prior to use; Brazilin stock was prepared in DMSO and diluted. Solutions were incubated for 5 min, and then spectrophotometric measurements were obtained at 517 nm. The percentage of free radical inhibition was calculated by using the following formula:

![Figure 6](https://dx.doi.org/10.1021/acsomega.0c00396)
Figure 7. Flow cytometry analysis of Brazilin at different concentrations against the apoptosis induced by Aβ (25–35). Cells were double-stained with PI and Annexin V-FITC and then analyzed by flow cytometry. The bar graph panel in (A) shows the death pathway on SH-SYSY induced by Aβ (25–35) in 50 μM. 7(B–I) Histograms of untreated cells, vehicle, 100 μM H2O2, 50 μM Aβ, and Brazilin treatment for 24 h; each bar represents a triplicate measurement, and error bars represent the mean values ± SD; differences were established with Student’s t-test with a P-value <0.05.

Figure 8. ThT fluorescence inhibition of the lysozyme aggregation by Brazilin 5 μM.

% of inhibition = \frac{\text{absorbance control} - \text{absorbance sample}}{\text{absorbance control}} \times 100\%

Cytotoxicity Assay. The cytotoxic potential was assayed by seeding ≥10,000 cells/well into a 96-well plate format. Consistently, the plates were incubated at 37 °C with 5% carbon dioxide with Braz (2.5 and 5.0 μM) and Aβ (25–35). Propidium iodide (PI) and Hoechst, at a final concentration of 1 μg/mL each, were added to each well 1 h prior to obtaining readings. Images were acquired in a live-cell mode using a multwell plate reader (IN-Cell 2000 automated microscopy system; GE Healthcare) equipped with a 10× objective and an IN-cell analyzer 2000 acquisition v4.0 software (GE Healthcare). Four contiguous fields with a montage of 2 × 2 were acquired per well and per fluorescence channel. Image capture and data analysis were translated to an IN-Cell analyzer workstation v3.7.2 software (GE Healthcare), which was used to realize data segmentation of the images (thus providing the region of interest and the cytotoxicity percentages of cell death per each well). Each of the experiments was repeated eight times.

Immunofluorescence and Protein Colocalization. After cells were pretreated for 24 h with Brazilin prior to Aβ (25–35) exposure, they were washed with PBST (phosphate-buffered saline + 0.1% v/v Tween-20 detergent) and fixed with 4% paraformaldehyde in PBS. Cells were then incubated with a blocking solution (5% normal goat serum (NGS) and 5% of PBS fetal bovine serum in PBS with 0.1% Tween-20) for 1 h on the shaker to eliminate unspecific binding of the antibodies. After three repeated washes, cells were incubated with primary antibody overnight at 4 °C diluted in 3% BSA in PBST. The secondary antibody, conjugated with an Alexa Fluor tag, 488, and Texas Red (goat antimouse, antirabbit, and DAPI), was added for 2 h at room temperature on the shaker. Images were captured using an LSM 700 confocal microscope, assisted with Zen 2009 software (Zeiss). For consistency, images were acquired at a 512-pixel resolution by utilizing a 488 nm laser with a laser power of 5.0, speed 7, averaging 8 and 1.0 Airy Units (AUs). Besides, no further adjustments were applied in brightness, contrast, or gamma settings.

Flow Cytometric Detection of Apoptotic and Necrosis Cells. To discern between apoptosis versus necrosis pathways, cells seeded in a 24-well plate were harvested, washed with cold PBS, and centrifuged. Then, cell pellets were gently resuspended by using 100 μL of the binding buffer added with Annexin V-FITC and PI reagents following the manufacturer’s instructions (Beckman Coulter). Subsequently, cells were incubated for 15 min on ice while protected from light, followed by the addition of ice-cold binding buffer to the

Table 1. Summary of Compounds and Concentrations Used

| figures | compound                  | concentration | experiment          |
|---------|---------------------------|---------------|---------------------|
| 1       | amyloid-β                 | 100 mM        | DLS analysis        |
| 2       | Brazilin                  | 5 μM, 2.5 μM  | DPPH analysis       |
| 3       | Brazilin, DMSO, H2O2      | 0.1, 1, 2.5, S, 7.5, 12.5, 25, 50, 100 μM, 2%, 100 μM | cytotoxicity       |
| 4       | amyloid-β, DMSO, H2O2     | 1, 5, 10, 20, 25, 30, 35, 50, 2%, 100 μM | cytotoxicity       |
| 5       | Brazilin and amyloid-β, DMSO, H2O2 | 0.1, 1, 2.5, 5 μM, 20 μM, 2%, H2O2 | cytotoxicity       |
| 6       | Brazilin and amyloid-β, DMSO, H2O2 | 0.1, 1, 2.5, 5 μM, 20 μM, 2%, H2O2 | immunocytochemistry |
| 7       | Brazilin and amyloid-β, DMSO, H2O2 | 2.5, 5 μM, 50 μM, 2%, H2O2 | apoptosis assay    |
| 8       | Brazilin and lysozyme     | 5 μM, 2 mg/mL | fluorescence inhibition |
suspension. The mixture prepared according to the manufacturer’s instructions (Beckman Coulter) was gently homogenized and immediately analyzed via flow cytometry (Galilio, Beckman Coulter) using the FL1 and FL2 detectors. The percentage of apoptotic cells is defined as the sum of both early and late stages of apoptosis, Annexin V-FITC positive. For each sample, approximately 10,000 events (cells) were collected and analyzed by using Kaluza software (Beckman Coulter).31,52

Fluorescence Inhibition of Lysozyme Fibrillation. Lysozyme aggregates were prepared by dissolving 139 μM (2 mg/mL) of lysozyme in 20 mM KH2PO4 with 3 M guanidine hydrochloride at a pH of 6.3, for a total volume of 1.8 mL, as previously described.51,52 Brazilin was prepared as previously mentioned, dissolved in DMSO at a concentration of 5 μM, and added to its respective vials. Vials were incubated for 5 h at 37 °C, at a constant agitation of 500 rpm. After 5 h, the formation of fibrils was observed. Samples were added to a Quartz cuvette, followed by the addition of 20 μM ThT and measured using a DM455 Spectrofluorimeter (On-Line Instrument System, Inc.). A 2 min scan was performed at a constant integration time (0.1 s) using an excitation and emission monochromator at 450 and 482 nm, respectively. All measurements were performed in triplicate (Table 1).

Statistical Analysis. All data were obtained in replicates to demonstrate the experimental viability and variability between samples; therefore, data presents the average with the corresponding standard deviation ± SD. Statistical analysis was performed by using a two-tailed paired Student’s t-test to demonstrate the statistical significance of variances between the samples and controls. To identify significant differences between groups, a P-value was calculated.

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G.H. and L.M. conceptualized, designed, and performed experiments; analyzed all of the data; and wrote the manuscript. A.V.-R. provided experimental designs, data analysis, and also technical help in the cytotoxicity, flow cytometric, and confocal microscopy experiments, as well as edits to the figures and manuscript. E.G. performed DLS and ThT assays. M.N. conceived the project and reviewed the data and manuscript.

Funding
This study was supported by funding from the Alzheimer’s Disease Research Foundation to M.N. In addition, M.N. would like to thank the College of Science (Research Enhancement Award) at UTEP and Holly and Dr Eddie Vazquez (The El Paso Pain Center) for their financial support. Finally, the authors would like to thank Les and Harriet Dodson Endowment and the President’s Office for financial support.

Notes
The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

The authors profusely thank Gladys Almodovar and the staff members of the Cellular Characterization and Biorepository Core Facility of the Border Biomedical Research Center at The University of Texas at El Paso (UTEP). This facility was supported by Grants # 2G12MD007592, SG12MD007592, and SU54MD007592 from the National Institute on Minority Health and Health Disparities (NIMHD), a component of the National Institutes of Health (NIH). Also, the authors appreciatively thank Jyoti Ahlawat for the excellent technical assistance.

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