Treatment of

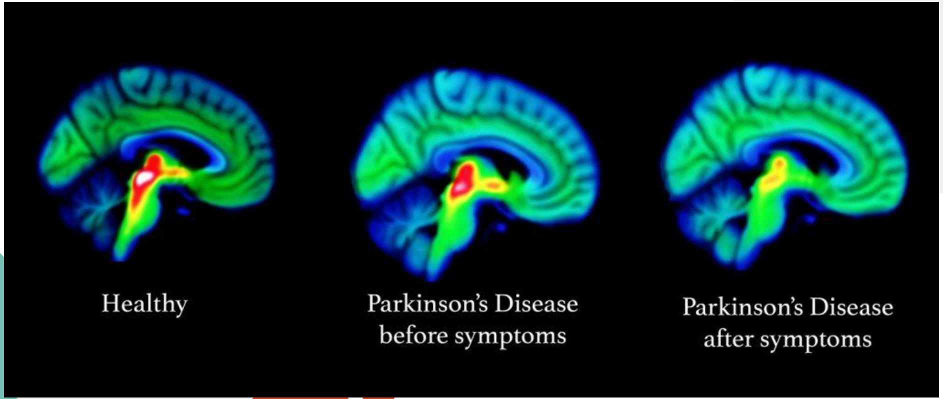
Neurodegenerative Diseases

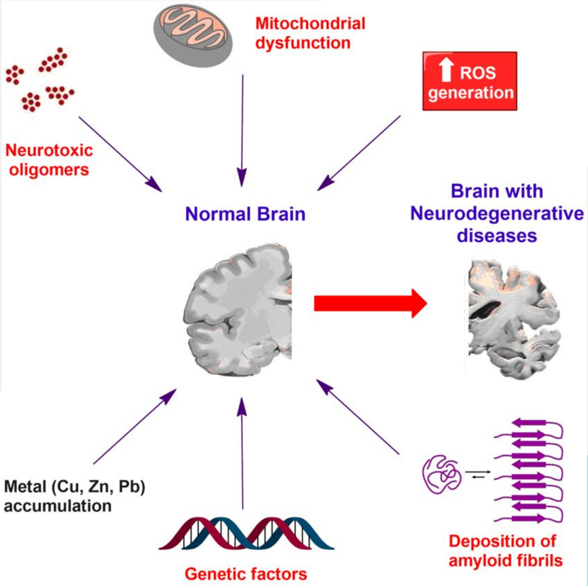
via Curcumin-Diethyl Fumarate Hybrid

Background Information

Neurodegenerative Diseases (NDDs)

- Loss of neurons in specific areas of the central nervous system, which leads to the progressive impairment of cognitive/motor functions
- Multifactorial ↔ No effective treatments

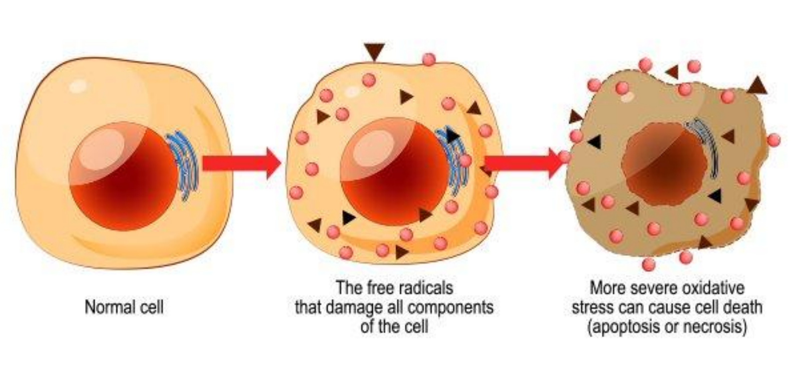




Oxidative Stress

Definition: imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive immediates or to repair the resulting damage

- Leads to misfolding of neurotoxic proteins
  - α-synuclein (α-syn)
  - phospho-tau (p-tau) and amyloid-β (Aβ)



Nuclear factor-erythroid related factor 2 (Nrf2)

- Important for antioxidant mechanisms in response to oxidative stress
  - Regulates the expression of detoxifying enzymes and antioxidant stress genes
  - Alleviates inflammation response

Altered expression of Nrf2 in neurons and astrocytes of Parkinson's and Alzheimer's patients

Why is this a problem?

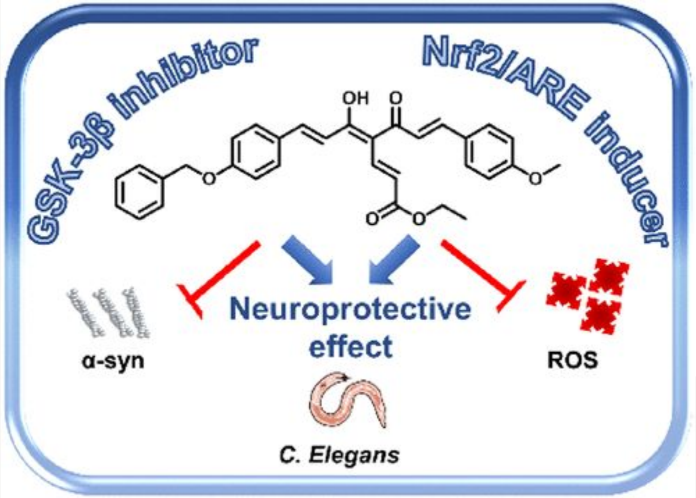
- Nrf2 activation reduces α-syn and p-tau levels
- Good defense against oxidative stress insults and loss of proteostasis

GSK-3β Enzyme

- Increased expression and activity in AD and PD
- Determining factor for abnormal tau protein phosphorylation and aggregation into neurofibrillary tangles
  - High levels of hyperphosphorylated tau ↔ high levels of insoluble α-syn
  - Leads to extensive oxidative stress and neuronal cell death
- Activated GSK-3β is connected to the downregulation of Nrf2
  - Negative correlation

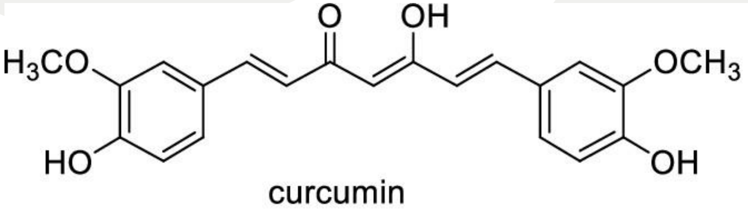
The Ideal Solution

- Inhibit GSK-3β and induce Nrf2 for optimal cell defense against oxidative stress
- Multitarget drugs are possible because GSK-3β and Nrf2 are both involved in the same signaling pathways in a feed-forward manner



Curcumin

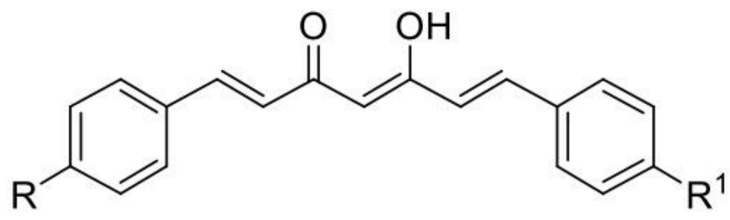
- Able to modulate many interconnected pathways implicated in the pathogenesis of multifactorial diseases like NDDs
- Capable of regulating mediators and biological targets of the inflammation cascade
- Considered as a pan-assay interfering compound (PAINS)
    - Can generate false assay signals
- Compound reactivity is dependent on structural context
- Molecules with a PAINS-based structural motif can act as selective modulators of well-defined targets



Discussion and Results

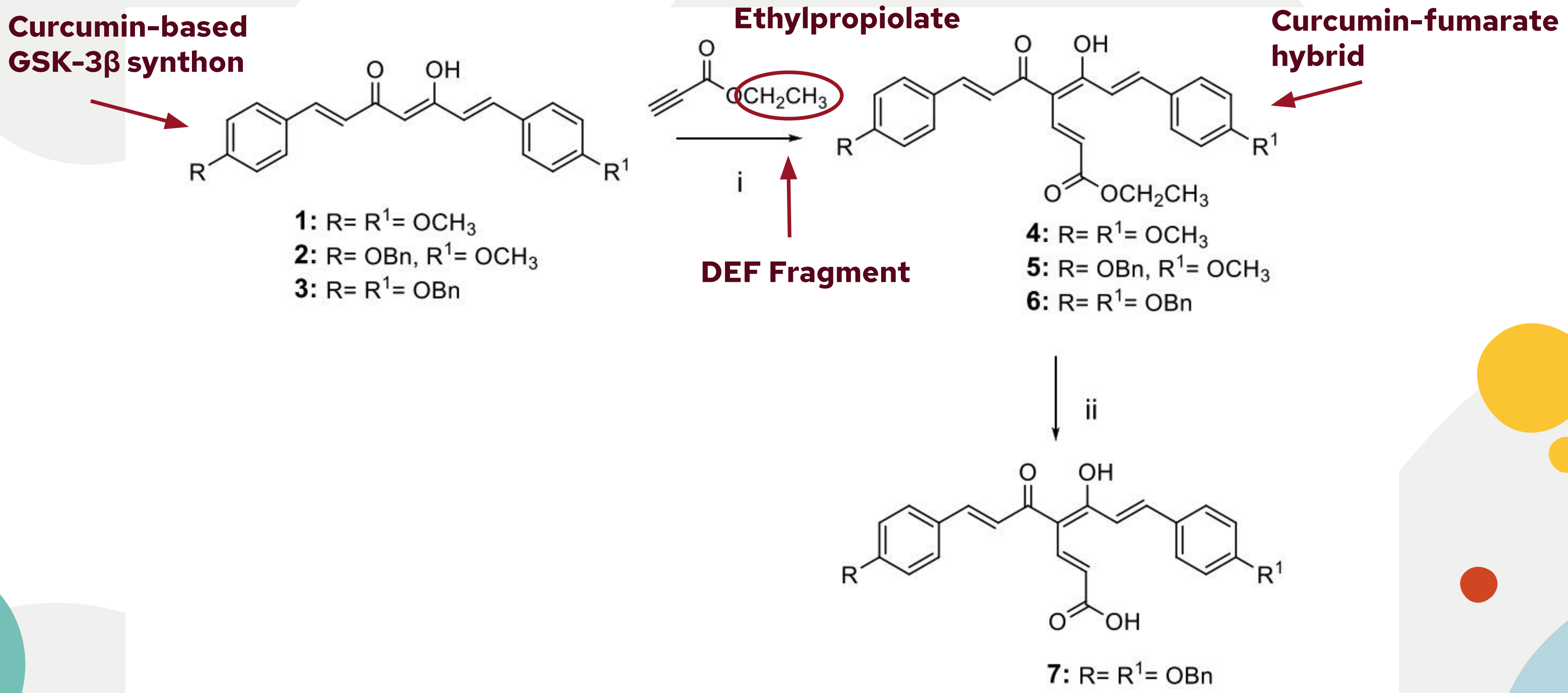
Design Strategy

- **Goal:** Concurrently modulate GSK-3β and Nrf2
- **Strategy:** Introduce a diethyl fumarate (DEF) fragment at the 4-position of the heptadienone framework of curcumin-based synthons
  - Identified GSK-3β inhibitors 1–3 serve as starting platform



curcumin-based GSK-3β synthons **(1-3)**

Synthesis of Curcumin-Fumarate Hybrids



Design of Study

Outlining Tests Performed on Drug

Overview

- Purpose
  - Ability to affect Nrf2 & GSK-3B activities
  - Protect neurons from damage by neurotoxins
- Types of Tests
  - Hybrids 4-7
  - Select Hybrids Based on Previous Tests
  - Hybrids 4-5

Tests Organization

1 Hybrids 4-7

1. **Measure GSK 3B Enzyme Inhibition**
   a. Luminescent Kinase Assay: amount of ATP post kinase reaction
   b. **5,6** → Mechanism of Inhibition
2. **Determine Ideal Concentration**
   a. MTT assay: neuron viability post-exposure to drug at range of concentrations
3. **Measure Indirect Antioxidant Activity**
   a. Probe: ⬇️ ROS formation in neurons post-exposure
   b. Probe: ⬆️ GSH levels as a cause of antioxidant activity
      i. **4,5** → GSH levels over time
4. **Measure BBB Permeation Ability**
   a. PAMPA-BBB Methodology: measure in vitro permeability

2 Select Hybrids Based on Previous Tests

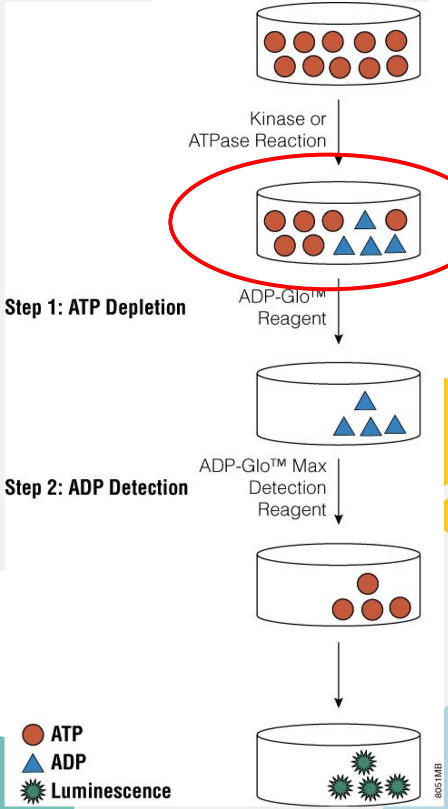
1. **Measure Effect on Nrf2/ARE pathway in 4-5**
   a. Western blot: cytoplasm-nuclear Nrf2 movement
   b. ELISA assay: Nrf2-ARE binding
   c. RT-PCR: NQO1 gene expression
2. **Measure ability to prevent neurotoxic effects in neuron 4-5**
   a. AD in vitro models: effect on B oligomers induced death
   b. PD in vitro models: effect on 6-OHDA induced death
      i. Fluorescence microscopy: A-syn aggregate formation
   c. In vivo C.elegans model: effect on 6-OHDA induced death in neurons in vivo

Tests Performed

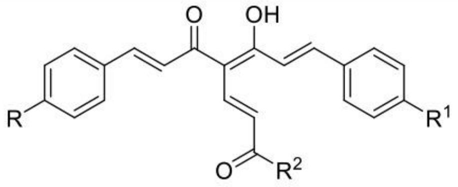
In Depth Discussion of Test Results

Biological Evaluations: Luminescent Kinase Assay

- Evaluate compounds' inhibition of the GSK-3β enzyme by using a human recombinant enzyme
- Based on the quantification of ATP present after kinase reaction
- First tested at highest concentration: 10 μM
  - IC_{50} value determined for derivatives showing an inhibition percentage > 50%



Biological Evaluations: Luminescent Kinase Assay

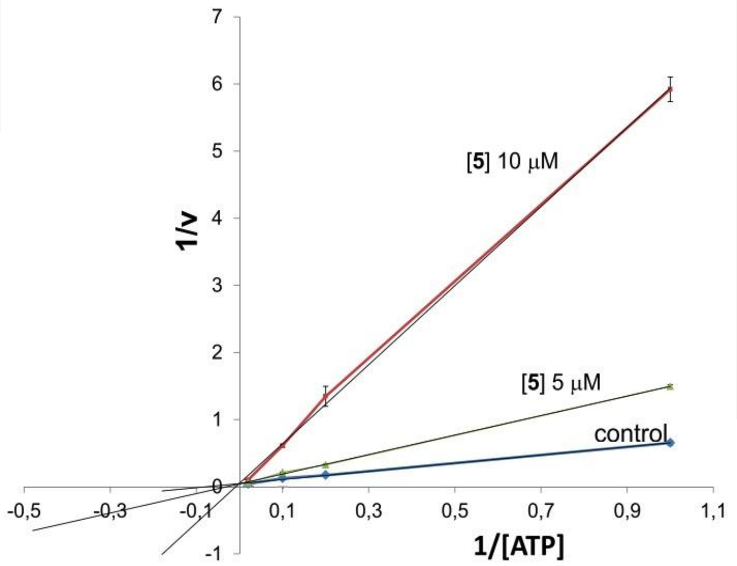
Compd				**GSK-3β inhibition**	
	R	**R^1**	**R^2**	**IC_{50} (μM)[a] ± SEM**	**inhibition (%)[a,b]**
4	OCH_3	OCH_3	OCH_2CH_3	>10	40 %
5	OBn	OCH_3	OCH_2CH_3	8.39 ± 0.34	
6	OBn	OBn	OCH_2CH_3	6.09 ± 0.53	
7	OBn	OBn	OH	>10	46 %

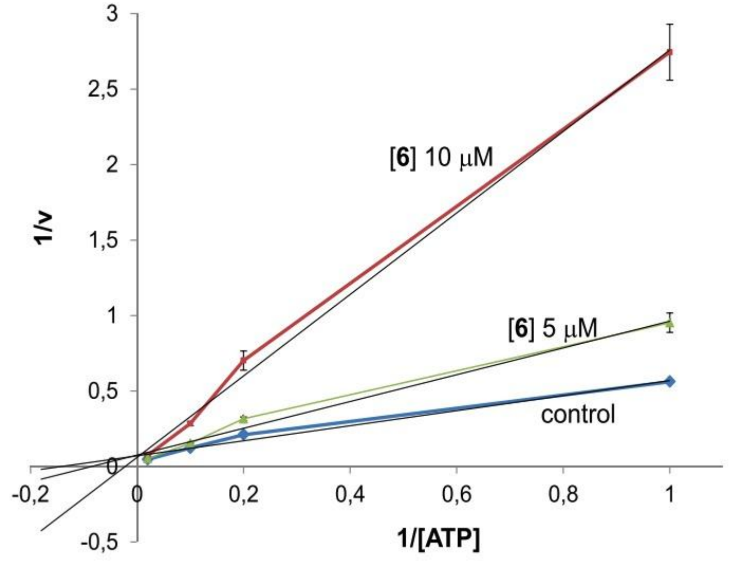
Smooth therapeutic inhibition of GSK-3□

Biological Evaluations: Kinetic Study

- Investigate the mechanism of inhibition as regards to competition with ATP for compounds **5** and **6**
  - Varying concentrations of ATP and tested compounds
  - Concentration of substrate kept constant

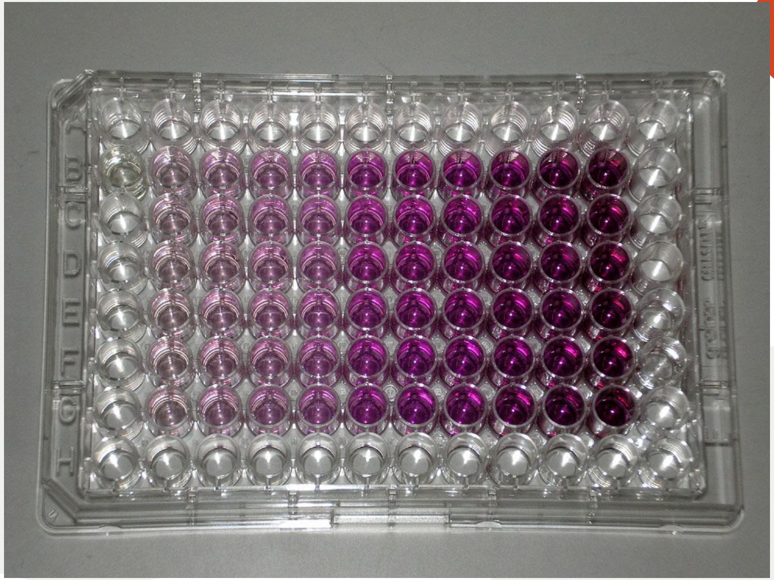
Biological Evaluations: Kinetic Study





Cytotoxicity: MTT Assay

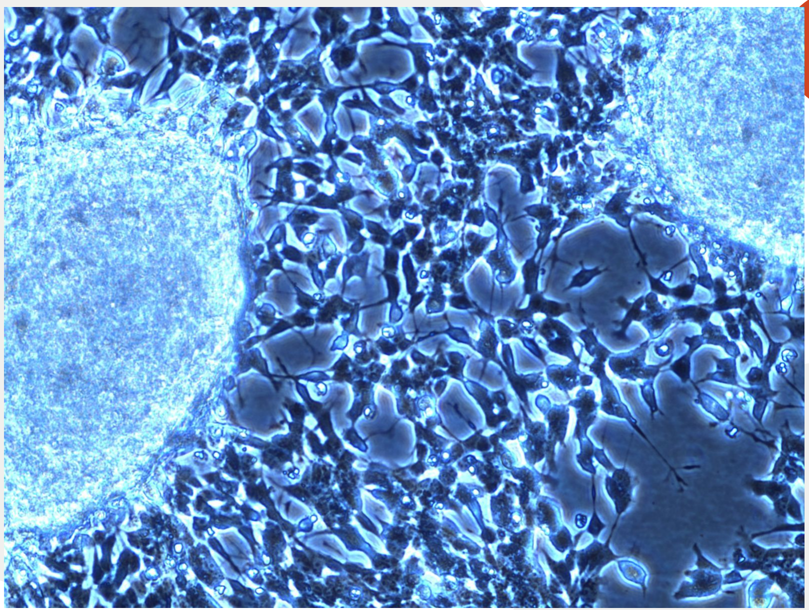
- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay
  - SH-SY5Y cells (human neuronal cells) exposed to varying concentrations (1.25-40 μM) of synthesized compounds



MTT Assay

Cytotoxicity: Findings

- All tested compounds at concentrations < 10 µM did not affect cell viability
  - Concentration of 5 µM selected to perform all following assays in SH-SY5Y cells



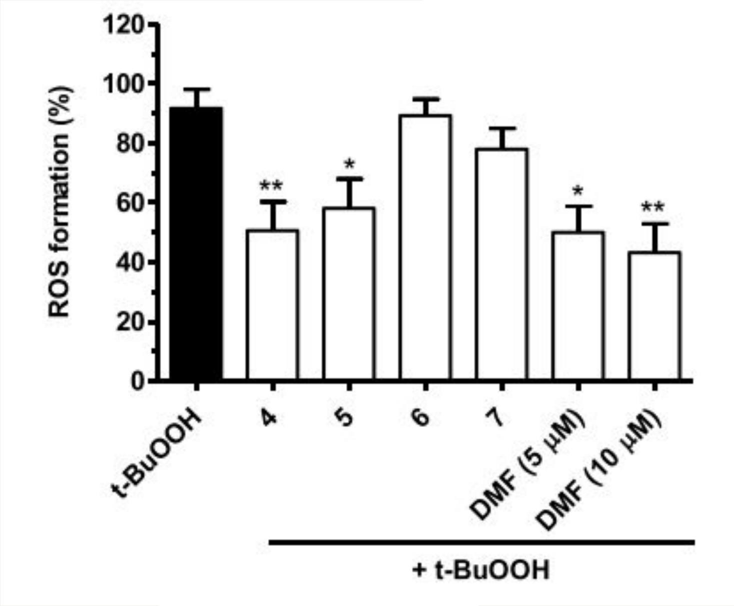
SH-SY5Y Cells

Antioxidant Activity

- Oxidative stress caused by reactive oxygen species (ROS)
- Antioxidants detoxify these radicals

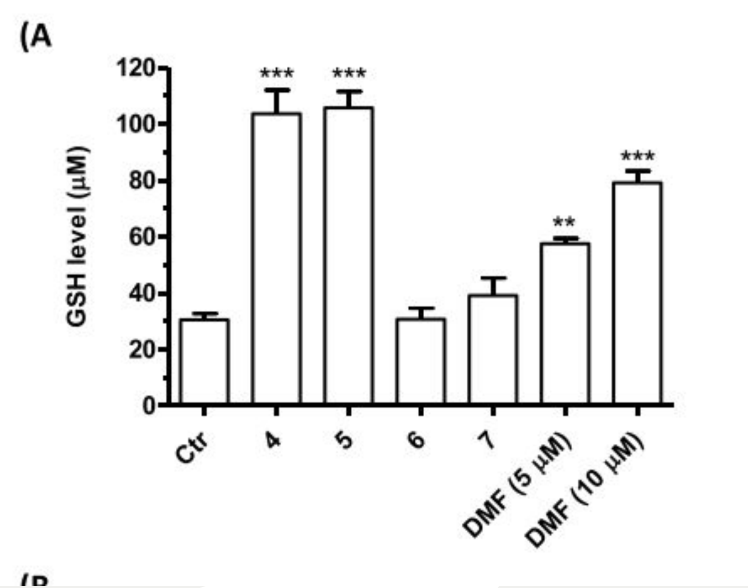
Antioxidant Activity

- Test compounds 4-7 as direct antioxidants
- Measure ROS levels (want them to decrease)
- Use fluorescent probe on ROS molecules



Antioxidant Activity

- Test how compounds 4-7 induce glutathione (GSH)
- Measure GSH levels (want them to increase)



Nrf2/ARE Pathway Activation

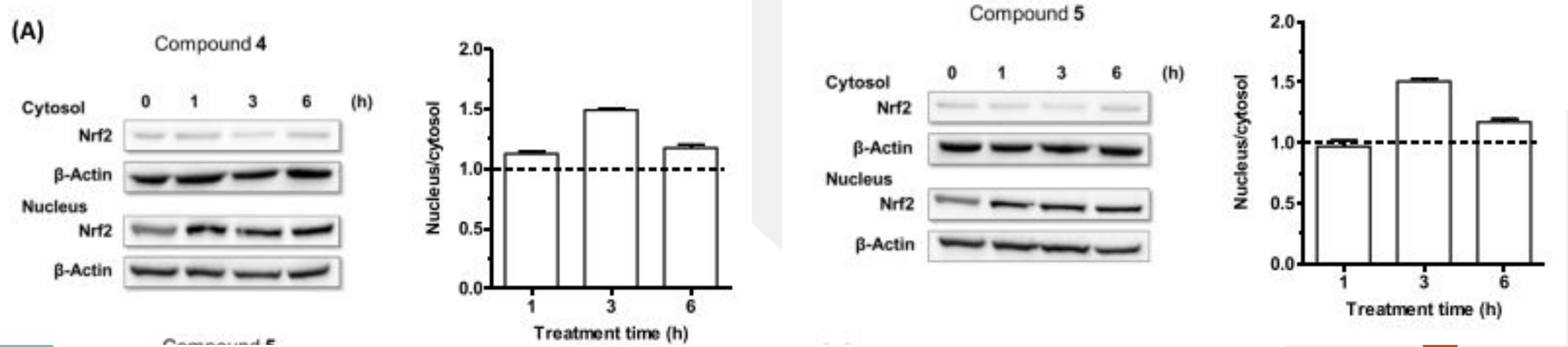
Oxidative stress and disrupted redox balance

⇒ Abnormal activity of the enzyme GSK-3β

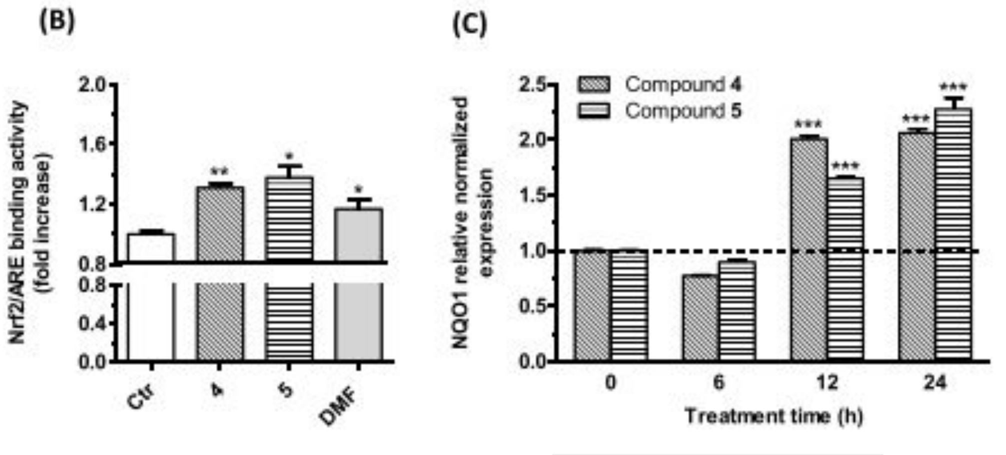
⇒ Impairment of the transcriptional activity of Nrf2

Nrf2/ARE Pathway Activation

- Track Nrf2/ARE pathways specifically
- Use Western blotting to quantify Nrf2 in nucleus and cytosol



Nrf2/ARE Pathway Activation

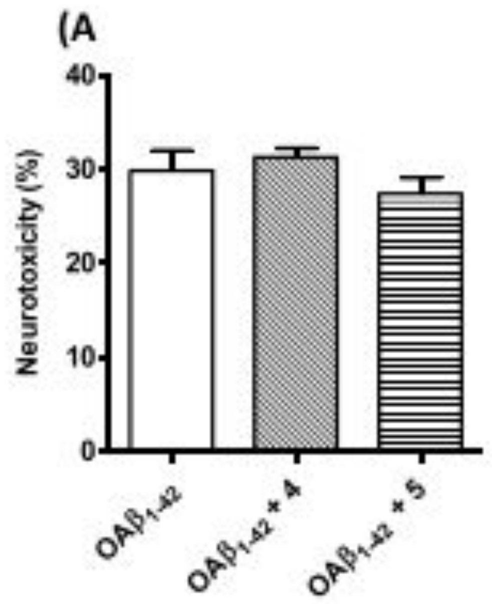


Neuroprotective Profile

- Test the neurotoxicity of compounds 4 & 5
- Ability to prevent SH-SY5Y cell death
- in vitro model of AD: accumulation of Aβ1−42 oligomers
- in vitro model of PD: induce cell damage with 6-OHDA

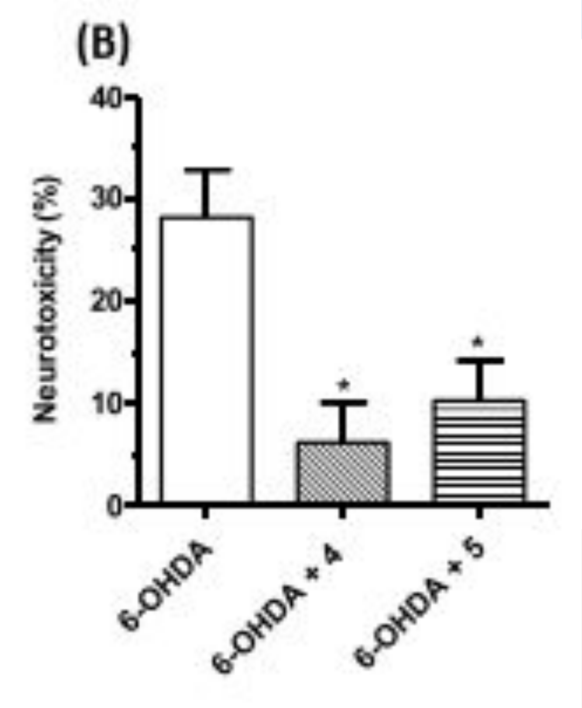
In Vitro Model of AD

- Neurotoxic effects from accumulation of soluble Aβ1–42 oligomers
- Both compounds failed to prevent Aβ1–42 oligomers induced cell death
- Incapability to serve as Aβ-based therapeutics

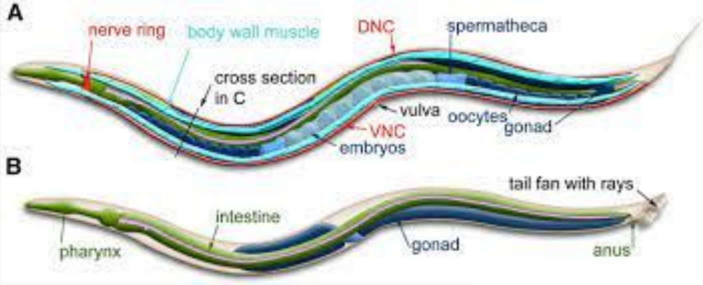


In Vitro Model of PD

- Neurotoxin 6-OHDA induces CNS oxidative damage and neuroinflammation
- Both compounds mitigate the 6-OHDA-induced decrease in cell viability
- Potential usefulness in the PD therapeutic area



Neuroprotective Effects: C. Elegans Model



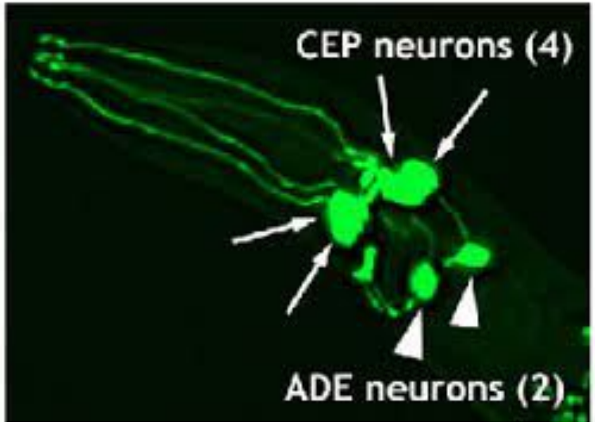
C. elegans Model - In vivo

- Short life cycle, fast reproduction, high genetic inheritance
- 8 DA neurons

- Expression of antioxidant genes relies exactly on same inhibition model in humans
  - GSK-3 inhibits SKN-1 → no antioxidant gene expression
  - Identification of neuroprotective agents that can inhibit GSK-3 is possible
- BY250 strain expresses GFP and can be visualized over time

Neuroprotective Effects: C. Elegans Model

- Investigations via this model will include:
  - Exposing nematode to PD-inducing toxin (6-OHDA)
  - Visualizing of GFP from DA neurons



Neuroprotective Effects: Methods

Incubated with 5 μM of each hybrid for 30 minutes

- With 5 μM of 6-OHDA
- After 72 hours, 4 CEP neurons examined and analyzed
- Nematodes incubated in 6-OHDA only → 68% degeneration

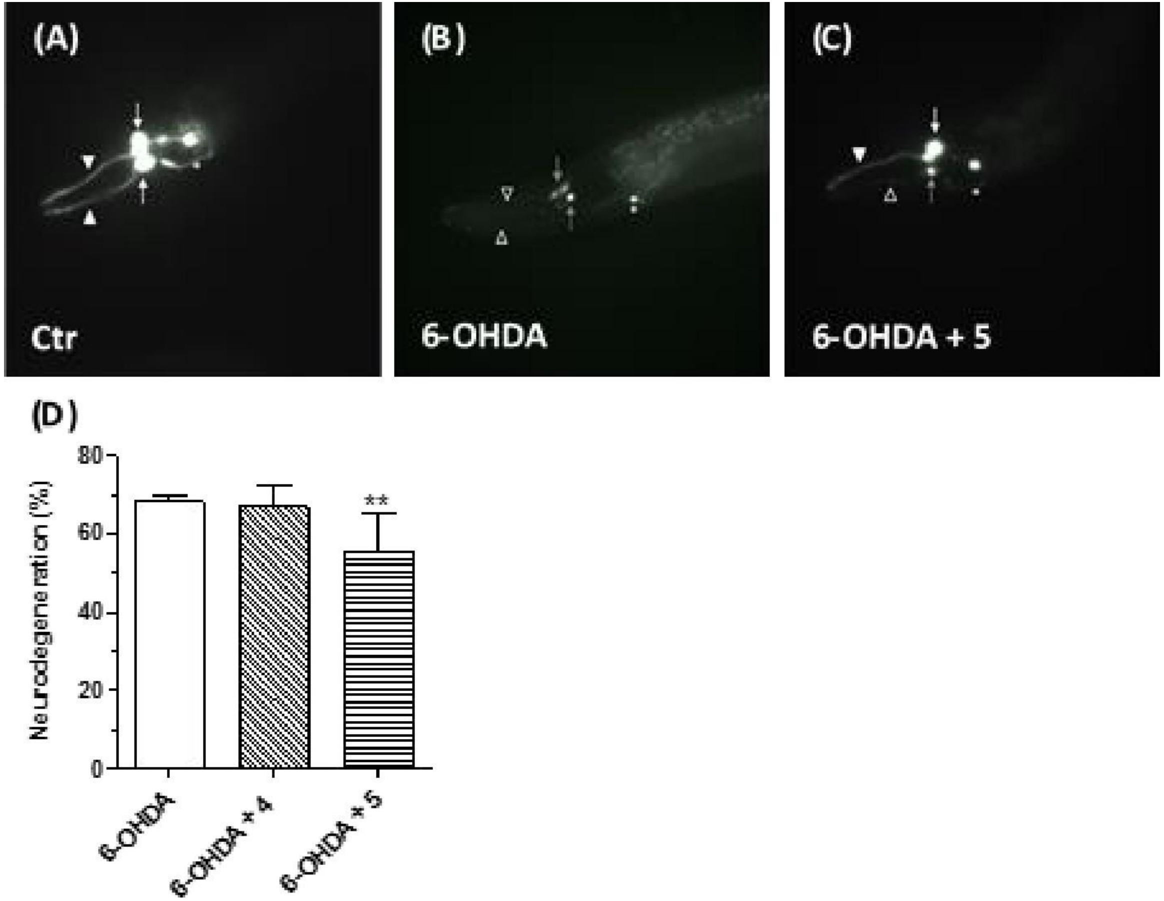
Neuroprotective Effects: Results

Curcumin-DEF Hybrid 4

- No phenotype salvaged
  - 67% of CEP neurons degenerated

Curcumin-DEF Hybrid 5

- Partial rescue of phenotype
  - 55% of CEP neurons degenerated


(A)
Ctr
(B)
6-OHDA
(C)
6-OHDA + 5
(D)
Neurodegeneration (%)
80
60
40
20
0
**
6-OHDA
6-OHDA + 4
6-OHDA + 5

35

Neuroprotective Effects: Conclusions

Analogue 5 proved to be the most effective protection against PD-inducing toxins

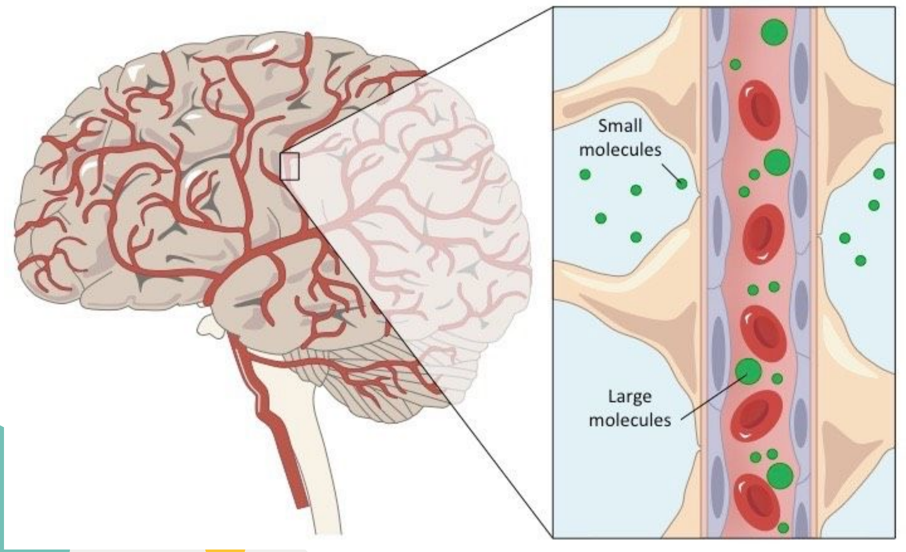
- 5 was the only effective inhibitor of GSK-3β
- Keap-1-dependent effects
    - Nrf2 may be released due to redox modification
- Activation of PI3K/AKT which leads to inhibition of GSK-3

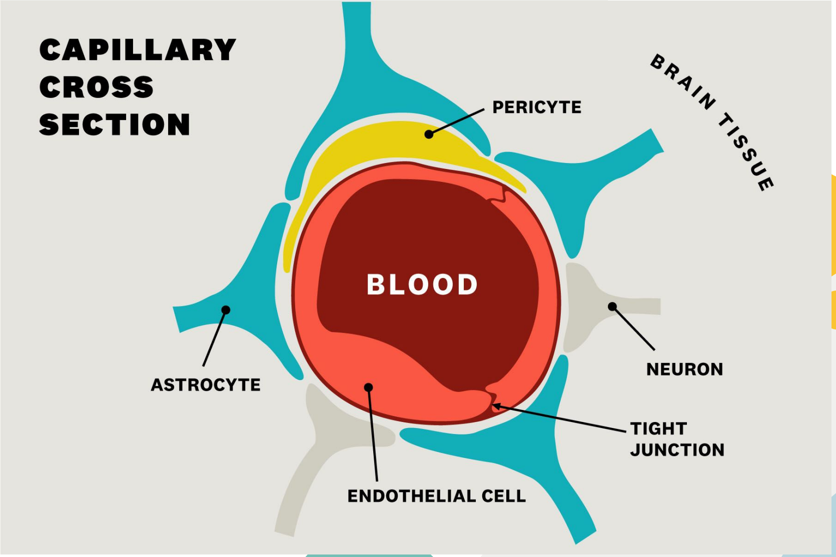
Further investigation showed compound 5 also effectively inhibited GSK-3β in SH-SY5Y cells

- Can suppress degeneration in neuronal cells as well

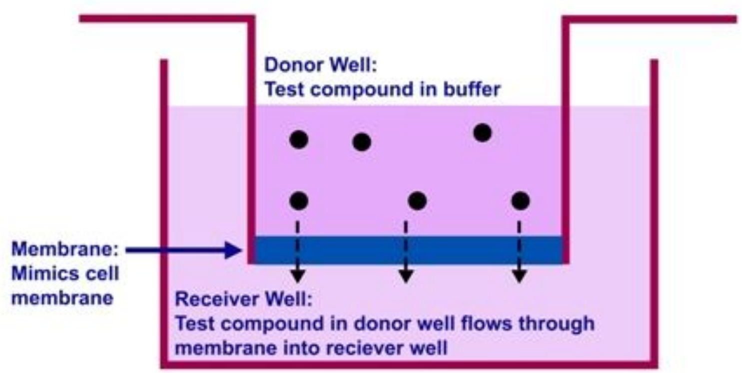
Blood-Brain Barrier Permeation

- Controls the capability of the drug to reach the central nervous system
  - Contributes to the effectiveness of NDD treatment

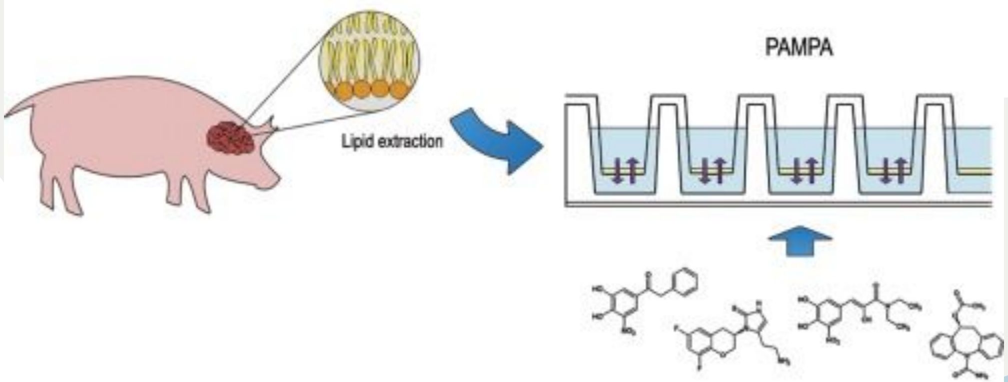




Blood-Brain Barrier Permeation



PAMPA: parallel artificial membrane permeability assay



Blood-Brain Barrier Permeation

- Compounds showing a Pe value superior to 2.26 × 10−6 cm s−1 should be able to permeate the brain compartment (CNS +)

Table 2. Permeability (Pe 10^{-6} cm s^{-1}) in the PAMPA-BBB Assay for Compounds 4−7 with Their Predictive Penetration in the CNS[a]

compound	Pe (10^{-6} cm s^{-1})[b]	prediction
4	2.5 ± 0.3	CNS +
5	4.8 ± 0.4	CNS +
6	4.5 ± 0.6	CNS +
7	1.7 ± 01	CNS +/CNS −

[a]The PBS:EtOH (70:30) mixture was used as solvent. [b]Data are the mean ± SD of two independent experiments.

Conclusion

Comparing Curcumin-Fumarate Hybrids

- Derivative 7 was not chemically stable; the formation of a degradation product was observed
- Regarding **GSK-3β inhibition**, derivatives 5 and 6 turned out to be the most effective
- Analogues 4 and 5 increased GSH intracellular levels through the **activation of the Nrf2/ARE pathway**
  - Capable of inducing Nrf2 nuclear translocation and intensifying Nrf2/ARE binding activity
- 4-5 emerged as dual GSK-3β/Nrf2 modulators and had **good BBB-penetrating capabilities**

Effectiveness of Compounds 4/5

- 4 and 5 demonstrated a protective effect against the **neurotoxicity induced by 6-OHDA**
- Compound 5 recorded a neuroprotective effect when observing PD induced transgenic **C. elegans** CEP dendrites/cell bodies
  - Partial **rescue of the toxic effects** induced by 6-OHDA
- **Multipotent profile** could represent a lead compound worthy of further development for disease-modifying PD therapeutics

Table of Results

curcumin dimethyl-fumarate compound	GSK-3β inhibition	antioxidant activity	Nrf2/ARE pathway activation	In vitro AD	In vitro PD	Neuroprotective in C.elegans	BBB permeation	stability
4	-	+	+	-	+	-	+	+
5	+	+	+	-	+	+	+	+
6	+	-	N/A	N/A	N/A	N/A	+	+
7	-	-	N/A	N/A	N/A	N/A	+/-	-

Shortcomings

- Only inhibited GSK-3β activity, when activation of GSK-3β was closely involved in Nrf2 regulation as well as oxidative stress defense
    - Common molecular pathogenic mechanisms including oxidative stress, proteostasis, **mitochondrial deficit**, **glutamate excitotoxicity**, and neuroinflammation
- Failed to treat Alzheimer's Disease
    - Did not prevent Aβ1−42 oligomers-induced cell death
- Still in **pre-clinical testing** stages as of 2020
    - Has not yet been tested for bioavailability, drug toxicity, or for method of drug delivery in humans (only animals)

Kahoot!

References

Di Martino, Rita Maria Concetta, et al. *Novel Curcumin-Diethyl Fumarate Hybrid as a Dualistic GSK-3β Inhibitor/Nrf2 Inducer for the Treatment of Parkinson's Disease*, Nov. 2020, pp. 1–13.