FINDING AND TESTING A POTENTIAL NOVEL TREATMENT FOR ALZHEIMER'S DISEASE

Examining the Neuroprotective Ability of N-Methyl-4-Isoleucine Cyclosporin in Amyloid-β (1-42) Neurotoxicity

Jeremy Kalfus

Indian Springs School; 11th Grade

Abstract

The amyloid beta (Aβ) peptide is the most notorious factor in the etiology of Alzheimer's disease (AD). The formation of amyloid beta plaques throughout the brain is synonymous with mass neuronal death, which leads to a major cognitive decline attributed to AD. A wealth of literature has attributed amyloid beta's neurotoxic abilities to the formation of mitochondrial permeability transition pores (mPTPs) in neurons, whereupon mitochondrial dysregulation leads to cell death.

In my project, I compiled a list of various potential compounds that were able to close mPTPs (via binding to a protein that regulates their opening named cyclophilin D). I then performed molecular docking simulations to find out which of these compounds was the best at binding to cyclophilin D, while still avoiding other cyclophilins in the body that may be important for functioning. The best compound was calculated to be NIM811, an analog of the drug cyclosporine A. To the best of my knowledge, neither NIM811 or CsA has published research in the context of any neurodegenerative disease.

I then cultured hippocampal rat neurons (hippocampal neurons are primarily affected in AD) and administered Aβ peptide to simulate AD, while treating the cells with 0, 5, or 20 µg of NIM811 as well. After a few days of incubation, I analyzed the cell's viability as well as mitochondrial permeability via flow cytometry. I found that NIM811 was able to close cells' mPTPs and improve their survival rates in the presence of Aβ.

Introduction

Alzheimer's Disease

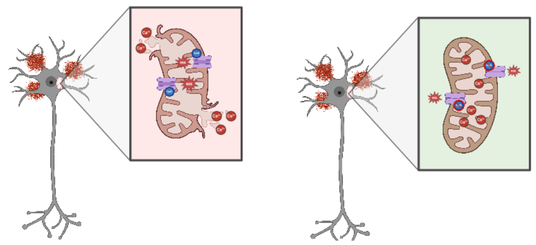
Alzheimer's disease (AD) is a neurodegenerative disease that primarily affects the elderly population. More than 70% of Americans over 75 suffer from it. Caused by massive neuronal death, symptoms include (sequentially): memory loss, severe cognitive dysfunction, and finally loss of life. Currently, there is no known method of cure or significant prevention for AD, only treatments that delay the inevitable.

Amyloid-β and how it kills neurons

Amyloid-β (Aβ) is a peptide that builds up in the brains of people with AD and is believed to cause it. While the etiology of AD slightly mysterious, it is largely believed that Aβ causes the neuronal degradation that leads to the classical cognitive decline associated with dementia. But how does Aβ actually kill neurons? Numerous studies have linked its effects to the disruption of mitochondrial permeability through the formation of mitochondrial membrane permeability pores, which damage the mitochondria and release reactive oxygen species (ROS) and calcium ions (Ca^{2+}) into the neuronal cytosol. Thus, the damaged mitochondria coupled with cellular stress from ROS and Ca^{2+} levels lead to brain cell death.

Research Objectives

Currently, every existing treatment for AD revolves around either improving memory (ChEI drug class) or stopping Aβ in very early stages (mAb drug class).

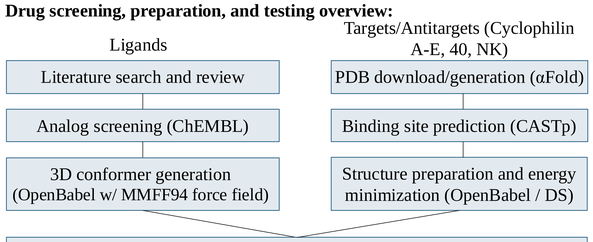


Neuron w/ open mPTPs in AD | **Neuron w/ closed mPTPs in AD**

However, it theoretically possible to treat AD in a third way, by stopping Aβ from killing neurons. Because Aβ likely exerts its neurotoxic effects by opening mPTPs, if one could close them again then they could stop neural death. The only known way to close mPTPs is through inhibiting Cyclophilin D, a protein that regulates mPTP opening. As such, Cyclophilin D is a potential drug target for treating AD.

Methodology

Drug screening, preparation, and testing overview:



RMSD is a metric for the distance between atoms of predicted vs. known structures. Average RMSD is a way of evaluating the accuracy of MD simulators

$$\mathrm{RMSD}(\mathbf{v},\mathbf{w}) = \sqrt{\frac{1}{n}\sum_{i=1}^{n}\|v_i - w_i\|^2} \ \text{Å}$$

Quantitative analysis of MD results and subsequent ranking:

Drug Name	CypD Binding Affinity (kD) DOCK (0.57)	Vina (0.55)	Diff (2.53)
A14	-8.6	-8.6	-8.6
Alisporvivir	-5.8	-5.9	-5.8
⋮			
Sang. A	-8.1	-8.3	-7.2

Where:

- r_i = Negative Model RMSD (b/c it's better smaller)
- d_i = Drug kD for respective simulator
- A_{CypD} = Calculated affinity score for CypD binding
- A_i = Each affinity score for binding to antitarget (Cyp A-C, E, 40, NK)

Mean binding affinity scored by weighed RMSDs

$$\vec{R} = \left\langle \frac{r_1}{r_1+r_2+r_3}, \frac{r_2}{r_1+r_2+r_3}, \frac{r_3}{r_1+r_2+r_3} \right\rangle$$

$$\bar{d} = \frac{1}{3}\sum_{n=1}^{3} d_i \qquad A = \bar{d}\sum_{i=1}^{3}\vec{R}_i$$

AutoDock Vina RMSD: 0.55 / R_1: 46%	UCSF Dock RMSD: 0.57 / R_2: 45%	DD 9%

Target affinity weighed against mean antitarget affinity

$$S = \frac{A_{CypD}}{\frac{1}{6}\sum_{i=1}^{6} A_i}$$

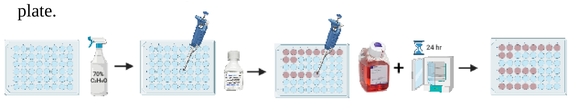
Final metric, used in ranking drugs

Effectively, this is the ratio of preference to CypD over other Cyclophilins

***In vitro* testing of NIM811:**

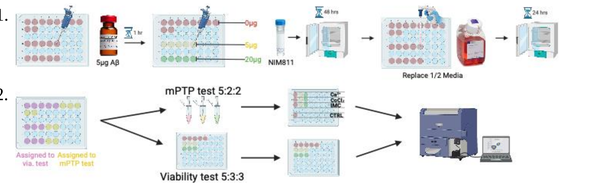
Preparing the neuronal cell culture:

- $2x10^6$ primary hippocampal rat neurons were obtained from Thermo Fisher Scientific
- 48-well plate was sterilized with 70% ethanol and then air dried
- After approx. 10 mins, plate was coated in 500 µl of 10 µg/ml Poly-l-lysine and incubated for 24 hrs. at 37°C in a 5% CO_2 humidified incubator
- After incubation, the wells were rinsed with 500 µl of PBS and 0.5 mL of neurobasal medium (including HBSS, 2% B27, 1% l-glutamine, and 1% penicillin–streptomycin) was emptied into the wells
- 100,000 neurons were plated in one line of 10 and two lines of 5 on the well plate.



Group assignment and testing

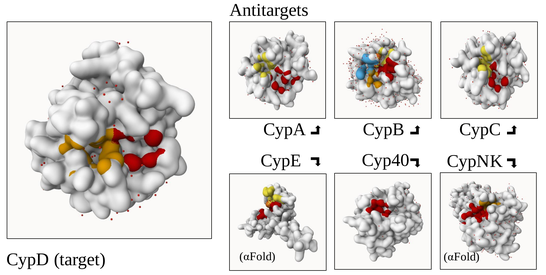
- Another 24 hrs. of incubation. Aβ powder dissolved in DMSO, sonicated, and aggregated; 5µg Aβ added to each well
- 3 treatments assigned: 0µg, 5µg, 20µg NIM811
- ½ serum media replaced after ~48 hrs.
- After 3 days: 5:2:2 wells used for mitochondrial permeability testing, dissociated into suspension. 5:3:3 moved to FC plate for viability testing
- mPTP testing cells placed on FC plate after being divided 3:3:3:1. Ca^{2+}, $CoCl_2$, and Ionomycin applied to one of each treatment group, with one control.



- A calcein-CoCl2 quenching assay was used for mitochondrial permeability analysis (in ΔΨm), measured by FC at 488nm (calcein florescence)
- Cell viability was measured by flow cytometry via Guava® ViaCount™ Reagent, measured at 532 nm (viability) and 488 nm (nucleation)
- Kruskal-Wallis Test applied to both data types

Results

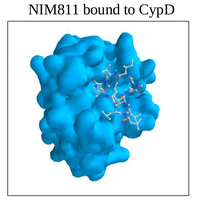
Binding Site Predictions (used in directing MD simulations)



Molecular Docking results (Only top 10 Final Score incl.)

	CypD Binding Affinity (kD) USCF DOCK	Autodock Vina	DiffDock
A14	-8.6	-8.6	-8.6
Alisporvivir	-5.8	-5.9	-5.8
Cyclosporine A	-5.7	-5.9	-6.4
Cyclosporine O	-5.1	-5.3	-5.1
Dimedone	-5.2	-5.2	-5.5
Gracillin	-6.8	-6.4	-6.6
Nim811	-9	-8.9	-9
Quinoloxazine	-5.9	-5.9	-5.7
Reconfilstat	-5.9	-5.9	-5.4
Sanglifehrin A	-8.1	-8.3	-7.2

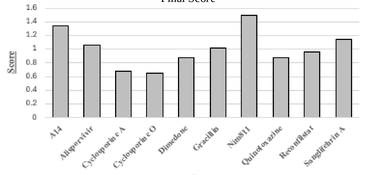
NIM811 bound to CypD



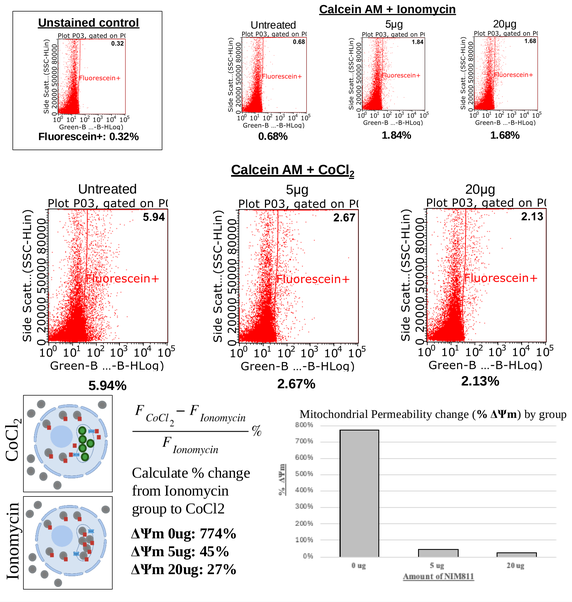
	CypA	CypB	CypC	CypD	CypE	Cyp40	CypNK
A14	-7.53	-6.05	-5.24	-8.6	-6.11	-7.24	-6.44
Alisporvivir	-6.88	-4.44	-5.2	-5.85	-5.53	-5.78	-5.49
Cyclosporine A	-9.65	-8.21	-8.74	-5.85	-8.38	-8.44	-8.55
Cyclosporine O	-8.32	-6.92	-8.33	-5.19	-7.8	-8.29	-7.85
Dimedone	-6.98	-5.66	-5.23	-5.23	-6.71	-6.02	-5.32
Gracillin	-5.74	-5.26	-6.73	-6.6	-7.55	-6.15	-7.56
Nim811	-6.69	-6.4	-5.98	-8.95	-5.35	-6.15	-5.46
Quinoloxazine	-8.07	-5.24	-5.89	-5.88	-7.35	-7.27	-6.63
Reconfilstat	-7.5	-5.32	-5.88	-5.86	-5.38	-6.84	-5.86
Sanglifehrin A	-6.4	-3.87	-4.12	-8.11	-9.18	-8.95	-9.98

Porportionally Weighted Avg of Binding Affinities

Final Score	
A14	1.336
Alisporvivir	1.053
Cyclosporine A	0.675
Cyclosporine O	0.655
Dimedone	0.874
Gracillin	1.016
Nim811	1.49
Quinoloxazine	0.872
Reconfilstat	0.956
Sanglifehrin A	1.145



Cell culture mitochondrial permeability test results



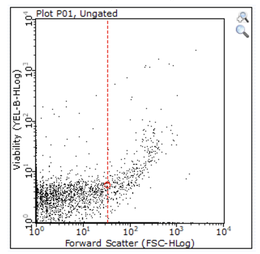
$$\frac{F_{CoCl_2} - F_{Ionomycin}}{F_{Ionomycin}} \%$$

Calculate % change from Ionomycin group to CoCl2

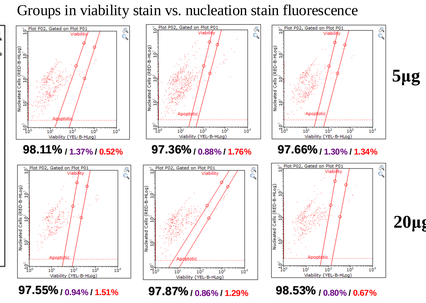
ΔΨm 0ug: 774%
ΔΨm 5ug: 45%
ΔΨm 20ug: 27%

Cell culture viability test results

Forward Scatter (event size) cutoff



Groups in viability stain vs. nucleation stain fluorescence



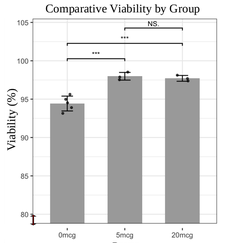
Group descriptive statistics

0µg group		5 µg group		20 µg group	
$\bar{x}$	94.43	$\bar{x}$	97.98	$\bar{x}$	97.71
σ	0.966	σ	0.500	σ	0.377
Q_1	93.91	Q_1	97.71	Q_1	97.51
Q_2	94.54	Q_2	97.87	Q_2	97.66
Q_3	94.97	Q_3	98.2	Q_3	97.89
n	5	n	3	n	3

Group samples ranked

0ug ranks	5ug ranks	20ug ranks
5	7	6
4	9	8
3	11	10
1		
2		

Comparative Viability by Group



Kruskal–Wallis Test (x-stand. ANOVA)

$$H = \frac{12}{n(n+1)}\sum\frac{R_i^2}{n_i} - 3(n+1)$$

Stat	0ug Group	5 ug Group	20 ug Group
r	15	27	24
n	5	3	3
r2/n	45	243	192

H=7.636
DF=2
p=0.022

Reject H_0

Stat	Value
n	11
K	3
H	7.636

Cross-group Mann–Whitney U test

$$U_1 = n_1 n_2 + \frac{n_1(n_1+1)}{2} - R_1$$

$$U_2 = n_1 n_2 + \frac{n_2(n_2+1)}{2} - R_2$$

Z score is just the Z of U score
"d" is effect size

	0ug-5ug	0ug-20ug	5ug-20ug
Z	-2.1002	-2.1002	0.3853
p	0.0357	0.0357	0.7
d	0.74	0.74	0.18
	reject H_0	reject H_0	don't reject

Discussion

- **NIM811 is likely a cyclophilin D inhibitor and shows promise in treating AD; of course, much more research is needed.** Multiple studies have argued evidence towards the former, this serves as confirmation
- **Cyclophilin D inhibitors are a promising new treatment class for AD and similar mitochondrial-related diseases. This study gave more evidence.**
- **This study was the first (that I could find) to employ a tri-platform molecular docking analysis**, as well as the first that weighted platforms based upon their predetermined accuracy. All three platforms used were complementary (they used different ways of determining kD).
- **This study was the first of its kind to test NIM811 in the context of any neurodegenerative disorder**, incl. AD.

Limitations

- **Untreated cell viability rates were significantly higher than in previous studies that employed in-vitro testing of Aβ**. I hypothesize that this is since I incubated the neurons-post infection for shorter time than most (3 days vs. 5-7 typical). This was because I could only stay in the lab for 4ish days. It could also be because I attempted to aggregate the Aβ into fibrils, as opposed to oligomerize it. Aβ oligomers have been shown to be more toxic *in vitro*.
- **Sample sizes were very small.** Most studies of similar caliber use ~48 or more neuronal wells, whereas this study only used 20. Furthermore, these 20 were divided into two testing groups This is because rat primary neurons are very expensive. The significance
- **This study does not provide causative insight into the role of mitochondrial permeability in apoptosis**
- **This study does not provide definite proof of Aβ's causation of mitochondrial permeability**.
- **This study used generative technology (AlphaFold) to simulate Cyp40 and CypNK. As such, MD results may be less accurate for these molecules.**
- **This study was done *in vitro*.** *In vivo* conditions are much more complex.

Key References And Citations

1. Trott, O., & Olson, A. J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. Journal of computational chemistry, 31(2), 455–461.
2. Corso, G., Stärk, H., Jing, B., Barzilay, R., & Jaakkola, T. (2022, October 4). DiffDock: diffusion steps, twists, and turns for molecular docking. arXiv.org.
3. Peter C Waldmeier, Jean-Jacques Feldtrauer, Ting Qian, and John J Lemasters. Inhibition of the mitochondrial permeability transition by the nonimmunosuppressive cyclosporin derivative nim811. Molecular pharmacology, 62(1):22–29, 2002.
4. Created in https://BioRender.com
5. Peter C Waldmeier, Jean-Jacques Feldtrauer, Ting Qian, and John J Lemasters. Inhibition of the mitochondrial permeability transition by the nonimmunosuppressive cyclosporin derivative nim811. Molecular pharmacology, 62(1):22–29, 2002.
6. Qinru Sun, Ning Jia, Weixi Wang, Hui Jin, Jiehua Xu, and Haitao Hu. Protective effects of astra-galoside iv against amyloid beta1-42 neurotoxicity by inhibiting the mitochondrial permeability transition pore opening. PloS one, 9(6):e98886, 2014.
7. Martin Tóth, John Hey, Aidan Power, and Susan Abushakra. Neurotoxic soluble amyloid oligomers drive alzheimer's pathogenesis and represent a clinically validated target for slowing disease progression. International journal of molecular sciences, 22(12):6355, 2021.