The effects of amyloid β-protein on the proliferation of human lung papillary adenocarcinoma

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Alzheimer's Disease (AD) is one of the most prevalent forms of dementia. Previous studies have shown Alzheimer's Disease is linked with an increase in the concentration of amyloid β-protein (Aβ); recent studies indicate an infection hypothesis could explain the presence and function of amyloid β-protein as an antimicrobial peptide with additional anti-cancer properties. In this study, the anticancer properties were measured by analyzing the effects of Aβ on the *in vitro* cell growth of papillary adenocarcinoma from the human lung tissue line (H441). Cells were cultured in varying concentrations of Aβ protein for a period of 48 hours under standard cell culturing conditions. The results yielded a significant ($p<0.0001$) inhibition of cell proliferation.

Abbreviations: AD – Alzheimer's Disease; Aβ – Amyloid β-protein; NTFs – Neurofibrillary tangles; AMP – Antimicrobial peptide; ACP – Anticancer Peptide; CHO – Chinese Hamster Ovary; PBS – Phosphate Buffered Saline; HDAC – Histone Deacetylase; TFA – Trifluoracetic acid

Keywords: Amyloid β Protein; Alzheimer's Disease; Infection hypothesis; Cell Proliferation; Anticancer; Antimicrobial

Introduction

Dementia is a set of persistent mental disorders in which an individual has marked changes in personality, memory, and impairment in reasoning. Alzheimer's disease (AD) is one of the most common types of dementia with a global prevalence close to 24 million, with future predictions to reach 50 million by 2032 (Mayeux and Stern, 2012). AD is pathologically delineated from the accumulation of amyloid β-protein (Aβ). The accumulation of Aβ forms β-amyloid plaques, neurofibrillary tangles of tau proteins, and causes inflammation (Moir et al., 2018). Fibrilization of Aβ appears to facilitate the defensive activities in the brain which leads to an increased concentration and collection of pathogens within the plaques of amyloid β-protein (Kumar and Moir, 2017). The varying stages of AD, both pre-clinical and clinical, have, for a long time, been explained predominantly by the amyloid hypothesis. This hypothesis suggests that the production of Aβ leads to a neuroinflammatory response and that this response, upon surpassing a threshold, results in AD (Fulop et al., 2018). The preeminent explanation of neuronal death from Aβ includes the disruption of plasma membranes via the production of pore-like structures (Moir et al., 2018).

Aβ has been shown to stimulate the innate immune system of organisms, after other microbial products have acted as stimuli to the brain and the periphery (Li et al., 2018). It is generated via the proteolytic cleavage of a larger precursor, and β-amyloid plaque formation is an innate immune response to what the body perceives as an immunochallenge (Moir et al., 2018). Recent studies have compared Aβ and the role it has as an antimicrobial peptide (AMP) to human cathelicidin antimicrobial peptide (LL-37), which is a representative AMP; these studies

have shown various similarities between the two peptides and have shown that Aβ, *in vitro*, can reduce the growth of pathogens by up to 200-fold. One possible explanation for the mechanism of action is that Aβ oligomers appear to facilitate the targeting of the carbohydrates in microbial cell walls. An additional possibility is the capture and trapping of microbes by Aβ fibrils in a protease-resistant structure of β-amyloid (Moir et al., 2018).

There has been a relatively recent increase in the acceptance of the antimicrobial protection hypothesis, also known as the infection hypothesis, which theorizes that an increase in microbial activity in the brain may aggravate the formation of β-amyloid plaques, leading to inflammation and AD advancement (Moir et al., 2018). The formation of Aβ fibrils activates neuroinflammatory pathways which aid in fighting an infection and eliminate β-amyloid aggregates. The constant activation of this response, as seen in AD, results in continued inflammation and neurodegeneration (Moir et at., 2018). The infection hypothesis stipulates that the formation of β-amyloid plaques may actually be a mechanism attempting to protect the brain from infection. *In vitro* tests have been conducted and show Aβ has potent microbicidal activity towards human pathogens. These findings are consistent with the identification of Aβ as an AMP (Kumar and Moir, 2017).

The discovery that Aβ has significant AMP properties against bacteria, fungi, and viruses, in concert with the evidence that AD neuroinflammation comes before Aβ deposition and the formation of amyloid β-protein plaques, increases confidence in an infection hypothesis (Fulop et al., 2018). Prior studies have shown that *Chlamydophila pneumonia* was found in patients with AD after their death. These studies have yielded results indicating systemic infection by this bacterium was associated with a 5-fold increase in the occurrence of AD (Balin et al., 2008). Additional investigations have found that herpes simplex virus type 1 (HSV-1) was involved in AD, and experiments showed that remnants of HSV-1 viral DNA were found in plaques (Itzhaki, 2014). Similar results were found with *Porphyromonas gingivalis* where it promoted Aβ deposition leading to the formation of β amyloid plaques. It was also found that Aβ has antiviral activity by creating pores in cellular membranes, resulting in the death of fungi, bacteria, and enveloped viruses (Fulop et al., 2018). Tests were conducted on mice by infecting them with pathogens and intracerebrally treating them with Aβ, resulting in an essentially total prevention of infection and plaque formation, suggesting the presence of physiological antimicrobial activity of Aβ. In an experiment where endothelial cells were infected with *Pseudomonas aeruginosa*, a gram-negative, rod shaped bacterium, Aβ was produced and released following the infection (Balczon et al., 2018). These experiments exhibiting that Aβ is an AMP have been vital in linking the infection and Aβ of AD (Fulop et al., 2018). AMPs typically kill infected or cancerous host cells, but unregulated AMPs can target healthy cells and are linked to disease as seen in AD. Aβ oligomers demonstrate the ability to target microbes but not all host cells (Moir et al., 2018).

Previous studies show many AMPs also share characteristics with anticancer peptides (ACPs). These shared properties make ACPs an active field of research for the use in future chemotherapeutic drugs (Felício et at., 2017). Cancer is the second leading cause of death worldwide behind cardiovascular disease. Current treatments of many cancers employ an array of chemotherapeutic methods which are related to the indiscriminate damage of normal cells. This pattern can lead to the development of multidrug resistance which suggests the need to discover more targeted, effective treatments (Pérez-Herrero and Fernández-Medarde, 2015). In 2020, it is predicted that there will be 1,806,590 new cases of cancer and 606,520 cancer related deaths in the United States (Siegel et al., 2020). In 2017, lung cancer was linked to more deaths than breast, prostate, colorectal, and brain cancers combined (Siegel et al., 2020). It is projected that in the United States from 2015-2065 there will be 4.4 million deaths related to cancer, 25% of them from lung cancer (Jeon et al., 2018). Due to the magnitude of cancer related deaths the development of cancer therapies is imperative. Chemotherapies that target both non-cancer and cancer cells of patients are some of the most widely used. The use of these treatments can lead to DNA damage and may be responsible for some of the long-term side effects of many cancer

treatments (Pich et al., 2019). For this reason, the development of immunotherapies is important to increase the positive outcomes for patients in the future. Immunotherapies enable the recognition of cancer cells as foreign by the host's immune system (Shroff et al., 2018).

NCI-H441 alpha cells are a cell line of papillary adenocarcinoma from human lung tissue. They are cultured largely via an adherent monolayer, although they can be counted floating or loosely attached to cell culture flasks. They are typically grown in modified Eagle medium with supplemented fetal bovine serum and cultured in 5-10% CO_2 at 37°C (Han et al., 2010). In this experiment, H441 cells were systematically cultured in varying concentrations of Aβ, and cell proliferation rates were measured to determine the anti-cancer properties of Aβ in reference to H441cells. Chinese Hamster Ovary (CHO) cells were used as a control.

Materials and Methods

Cell Culturing

Cell cultures of H441 and CHO cells were performed in 75cm^3 flasks. The base medium used was DMEM/F-12 50/50, 1X (Dulbecco's Mod. of Eagle's Medium / Ham's F-12 50/50 Mix) with L-glutamine & 15mM HEPES with 10% by volume fetal bovine serum which was warmed to approximately 37°C for 15 minutes prior to usage. All cells were routinely passaged as monolayers at 37 °C in a humidified environment of 5% CO_2 and 95% air until approximately 80% confluency was reached (Funamizu et at., 2012). The media was removed and discarded. Cells were then washed with phosphate buffered saline (PBS). 2.0 mL of Trypsin-EDTA solution was then added to the cell culture flask. The cells were then placed in an incubator at 37 °C to facilitate detachment for <4 minutes. Approximately 5.0 mL of growth media was then added and cells were gently aspirated using a pipette. Aliquots of cell suspension were added to new culture flasks with a sub-cultivation ratio of 1:10 (Morten et al., 2016).

When sub-culturing both the H441 and the CHO cells from the 75-cm^3 into the 96-well plate, 50 µL of cells in media were added to each of the experimental well plates according to protocol established by the American Type Culture Collection (ATCC, 2019). A protein solution of 0.1 mg of Aβ diluted in 5 mL of growth media was made. Aliquots were placed in 1 mL centrifuge filter tubes and spun to purify the sample. A 96-well plate was used for the experimental cell culturing. For each trial, 24 wells in each plate were used to culture the cells. A total of 24 different trials were conducted at four different concentration levels: 0.00 µM, 0.50 µM, 1.00 µM, and 2.00 µM. Each concentration level is tested with both H441 and CHO cells, which served as a mammalian control sample. Each trial is made up of 100 individual cell concentration counts. For the trials with 2.00 µM Aβ, 45.05 µL of the hydrated protein and 54.95 µL of media were added. For the trials with 1.00 µM, 22.52 µL of Aβ protein solution and 77.48 µL of media were added. For the trials with 0.50 µM, 11.26 µL of Aβ protein solution and 88.74 µL of media were added. For the control trials with 0.00 µM Aβ, 100 µL of media were added.

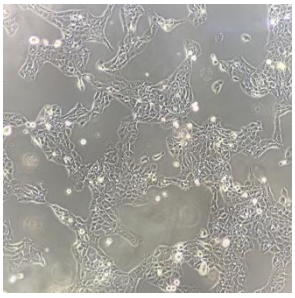

Figure 1: Image of a CHO cell culture prior to reaching the appropriate confluency. Image was captured at a magnification of x100.

Cell Counting

Growth media and trypsin were both pre-warmed at approximately 37 °C in a StableTemp digital warmer for 10-15 minutes. The solution was removed and placed in a 4 mL sample cup. Then, 100 µL of trypsin was placed in each of the 24 wells for approximately two minutes. Trypsin and cell solution were removed and added to the 4 mL sample cup. 100 µL of media were added with a micropipette, and remaining cells were aspirated three times. They were then removed and placed in the sample cup. 1 mL of media was

then added to the sample cup, bringing the total volume to 1.30 mL, resulting in a dilution factor of 13. A Vi-CELL XR Cell Counter was disinfected, primed, and then used to measure the cell concentration. Cell culture samples were logged and queued in the cell counter after selecting the appropriate programmed measurement parameters for H441 and CHO cell analysis.

Experimental trials

In prior experiments testing cell viability of Histone Deacetylase (HDAC) inhibitors, it was found that cell proliferation of H441 cells was suppressed at a concentration of 10 µM and lower (Han et al., 2010). In this study, the effects of Aβ on proliferation were measured at 2.00 µM, 1.00 µM, and 0.50 µM. The concentration 0.00 µM Aβ of cell growth media was used as a proteinaceous control. A control test was performed with a 96 well-plate to measure the growth rate of both cell strains to confirm when approximately 80% confluency would be reached. A large portion of cell growth media during this trial evaporated and lead to a decrease in cell growth. To prevent this, approximately 300 µL of cell growth media was placed in the outermost well plates to mitigate evaporation of media in experimental well plates.The Aβ protein that was used in the experimental trials contained 13% (by mass) trifluroacetic acid (TFA). To control for the possible effects that the TFA could have on the change in cell proliferation seen in the Aβ trials, H441 cells were cultured in a 96-well plate following the same protocols used in the Aβ experimental trials. H441 cells were cultured in solutions with 3.0 µM, 0.3 µM, 0.03 µM, and 0.00 µM of TFA and incubated for a period of 48 hours. For each concentration of TFA, 10 well cultures were performed. This same experimental set up and TFA growth conditions were followed for the CHO cell samples in a separate 96-well plate.

For all cell culturing in this experiment, cell cultures and 96-well plates were placed in an incubator for 48 hours. The incubator is programmed and design to maintain a stable incubating temperature of 37 °C, 5% CO_2, and maintain a high humidity via an internal water reservoir. All cell cultures were placed in an incubator at 37 °C and 5% CO_2 for 48 hours. These conditions, are used to optimize the growth of subcultures of adherent cells during cell culture processes (Ricardo and Phelan, 2008).

Results

The goal of the present study is to determine the effects of Aβ on cell growth *in vitro* of H441 cells. The results indicate that Aβ is a significant inhibitor of cell proliferation in H441 cell cultures. The cell concentration for the individual cells are seen in Table 1. Images A and B are taken from cell cultures of H441 at 0.00 µM and 2.00 µM, respectively. Arrows in Image B show a visual decrease in cell proliferation as seen at x200 magnification. The Vi-CELL XR counted the cell concentration 100 times for each of the 24 well plates within each of the experimental trials. The average cell concentrations ($x10^6$ cells/mL) for the H441 cells cultured in Aβ 0.00 µM, 0.50 µM, 1.00 µM, and 2.00 µM are 2.82, 1.58, 1.61, and 1.19, respectively (see Table 1). The average cell concentrations ($x10^6$ cells/mL) for the CHO cells cultured in Aβ 0.00 µM, 0.50 µM, 1.00 µM, and 2.00 µM are 1.34, 1.09, 0.60, and 0.79 respectively**.** The average cell concentration for H441 cells cultured in 2 µM Aβ is 1.19 x 10^6 cells/mL. The average concentrations for H441 cells cultured in 1.00, 0.50, and 0.00 µM Aβ are 1.61, 1.57, and 2.82 x 10^6 cells/mL, respectively. The average cell concentration for CHO cells cultured in 2 µM Aβ is 0.79 x 10^6 cells/mL. The average concentrations for CHO cells cultured in 1.00, 0.50, and 0.00 µM Aβ are 0.60, 1.09, and 1.34 x 10^6 cells/mL, respectively.

The values shown in Table 1 are the average of 100 cell concentration counts. The average cell concentration of H441 and CHO cells after 48-hour growth of 2.00 µM, 1.00 µM, 0.50 µM and 0.00 µM Aβ can be seen in Figure 2 A and B, respectively. Figure 2 shows the average cell count per well for cells cultured with Aβ at the concentrations of 2.00 µM, 1.00 µM, 0.50 µM, and 0.00 µM serving as a control. The standard errors for these counts are also displayed.

The values that are recorded above in Table 1 are plotted below in Figure 3 A and B,

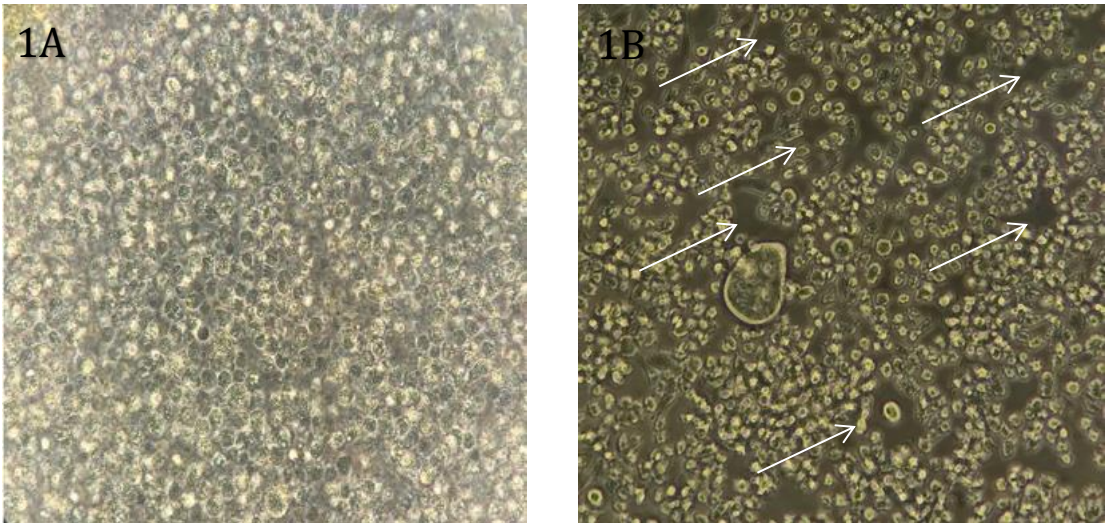

Figure 1A, 1B*:* Shown above are images of the cell growth for the cell cultures of H441 in 0.0 µM (A) and H441 cells in 2.0 µM cells (B). Images taken with an Olympus CKX53 at x200 magnification are shown. Areas of decreased cell proliferation are indicated by arrows.

Table 1. H441 and CHO Cell Cultures

Sample #	H441 [0.0 µM]	H441 [0.50 µM]	H441 [1.00 µM]	H441 [2.00 µM]	CHO [0.0 µM]	CHO [0.50 µM]	CHO [1.00 µM]	CHO [2.00 µM]
1	2.98	1.42	1.53	1.02	0.83	0.68	0.62	1.12
2	3.98	1.27	3.27	0.87	1.07	0.73	0.66	1.33
3	3.37	0.91	3.26	1.33	1.45	0.91	0.51	1.18
4	1.16	2.38	3.36	0.72	2.48	0.77	0.80	0.68
5	0.86	1.66	2.80	1.25	1.75	0.42	0.51	0.82
6	1.62	1.84	2.57	1.16	1.31	1.68	0.50	0.53
7	1.34	1.03	1.66	1.08	0.84	0.74	0.47	0.43
8	1.25	1.42	2.21	1.64	2.03	0.59	0.85	0.91
9	1.89	2.09	1.52	1.80	1.31	1.00	0.61	0.59
10	3.23	2.31	1.17	1.33	1.31	0.47	0.56	0.47
11	2.74	1.86	0.92	1.13	1.23	0.76	0.79	1.18
12	2.91	2.82	1.43	1.44	1.03	0.56	0.76	0.91
13	2.38	0.77	0.91	1.30	0.76	3.24	1.13	0.63
14	3.49	0.83	1.01	0.94	0.99	1.69	0.55	0.44
15	3.08	3.35	0.70	1.02	1.30	1.54	0.50	1.91
16	3.05	1.98	1.42	1.06	0.95	2.01	0.49	0.71
17	4.77	0.84	1.23		1.26	2.11	0.73	0.57
18	3.92	1.88	1.44		1.32	1.26	0.39	0.55
19	3.80	0.83	1.07		2.14	0.20	0.39	0.67
20	4.02	1.67	1.14		1.26	1.21	0.55	0.56
21	0.89	0.90	1.10		1.97	1.25	0.51	0.74
22	4.16	0.98	1.25		1.04	0.52	0.35	0.66
23	3.36	0.83	1.00		1.10	0.71	0.53	0.53
24	3.48	1.94	0.61		1.32			

Note. H441 and CHO cells are cultured with Aβ at four different concentrations: 2.00 µM, 1.00 µM, and 0.50 µM. There is also a sample cultured with 0.00 µM of Aβ which functions as a control. The average cell concentrations ($x10^6$ cells/mL) for the H441 cells cultured in Aβ 0.00 µM, 0.50 µM, 1.00 µM, and 2.00 µM are 2.82, 1.58, 1.61, and 1.19, respectively. The average cell concentrations ($x10^6$ cells/mL) for the CHO cells cultured in Aβ 0.00 µM, 0.50 µM, 1.00 µM, and 2.00 µM are 1.34, 1.09, 0.60, and 0.79, respectively.

2A

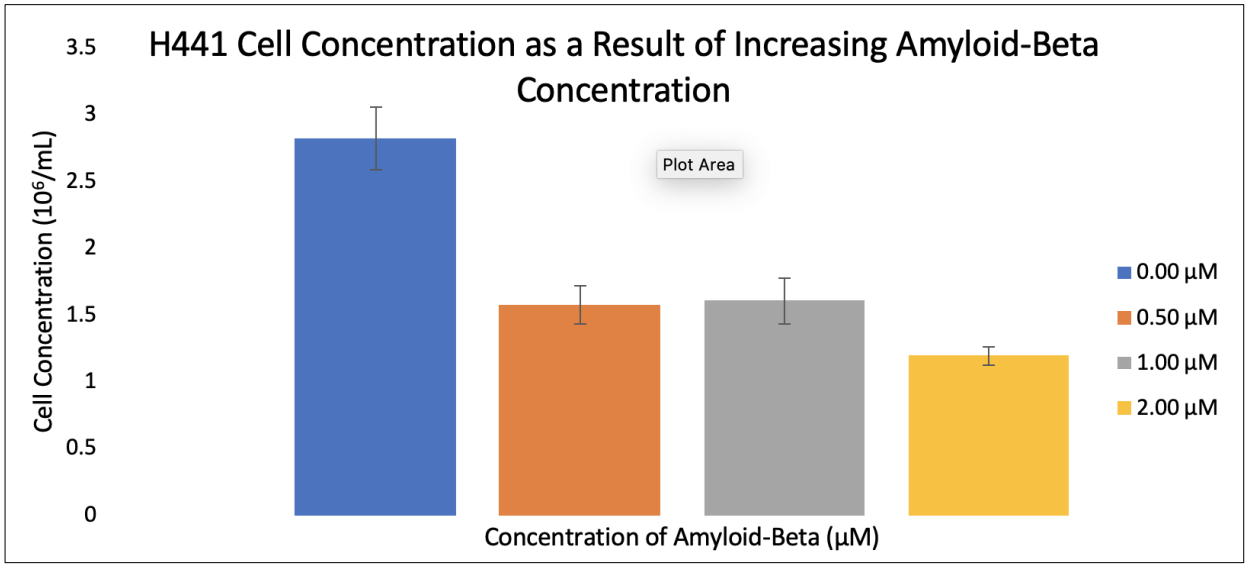

Figure 2A: The cell concentration for H441 cells in each trial are displayed in the figure above. The data for four different concentrations of Aβ are shown: 2 µM, 1 µM, and 0.5 µM. There is also a sample cultured with 0.00 µM Aβ which serves as a control.

2B

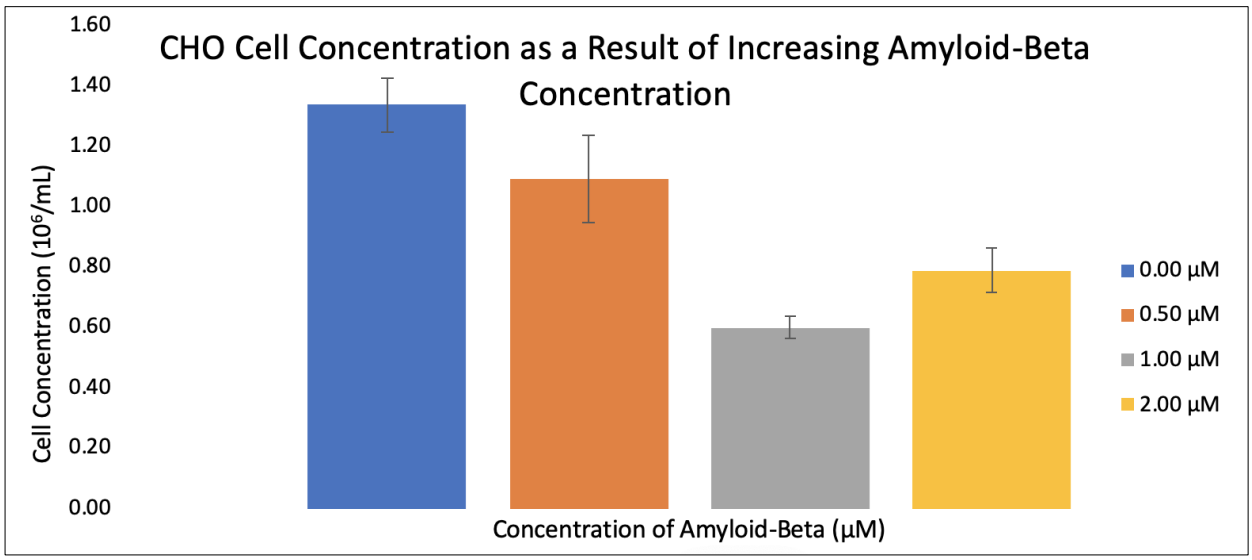

Figure 2B: The cell concentration for CHO cells in each trial are displayed in the figure above. The data for four different concentrations of Aβ are shown: 2 µM, 1 µM, and 0.5 µM. There is also a sample cultured with 0.00 µM Aβ which serves as a control.

3A

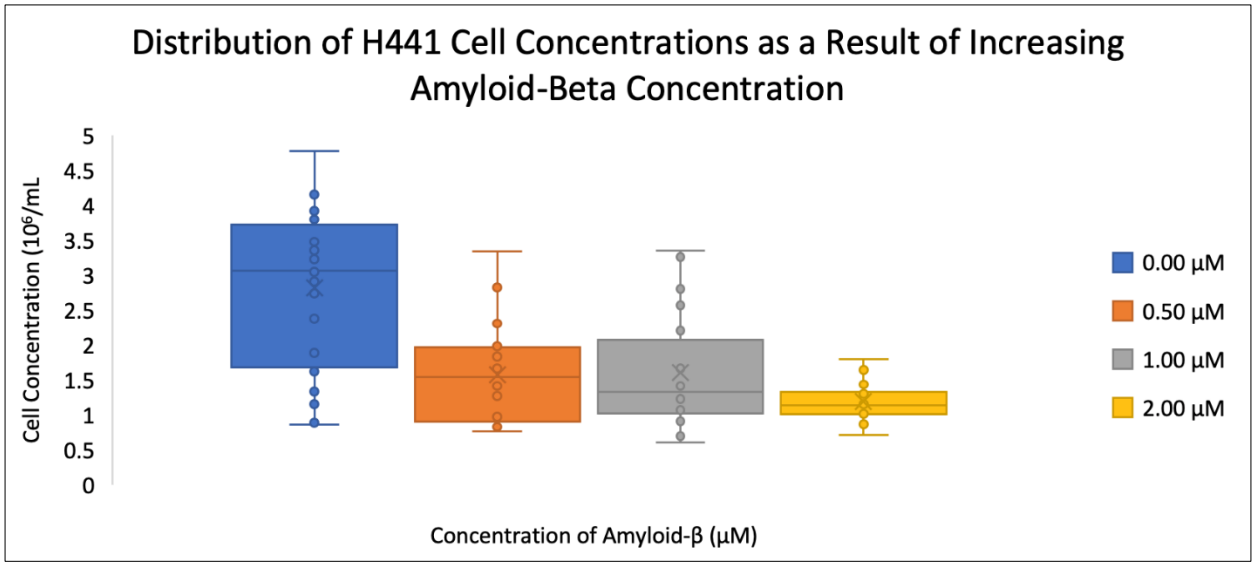

Figure 3A: Each of the four trials with different concentrations of Aβ are plotted against the cell count per well. Shown above is the data for the H441 cells. The standard errors for each of cell count per well in each concentration is also shown.

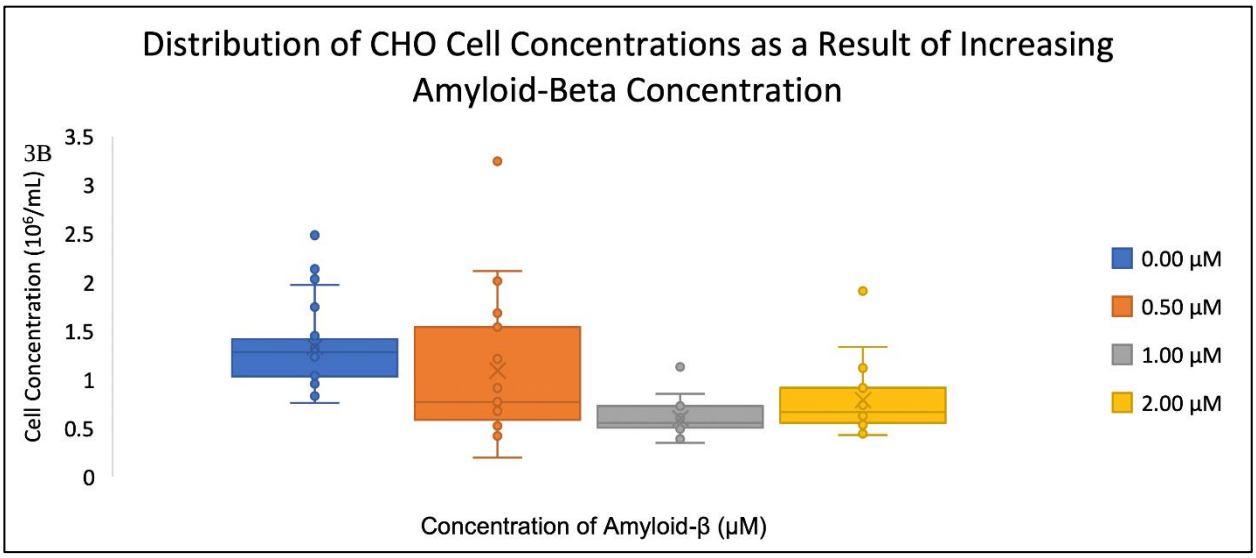

Figure 3B: Each of the four trials with different concentrations of Aβ are plotted against the cell count per well. Shown above is the data for the CHO cells. The standard errors for each of cell count per well in each concentration is also shown.

showing the spread of the data for the different concentrations for H441 and CHO cells, respectively.

The average decrease in cell proliferation, as compared to the control sample for H441 cells is 58%, 43%, and 44% for cultures in 2.00 µM, 1.00 µM, 0.50 µM Aβ, respectively. The average decrease in cell proliferation, as compared to the control sample for CHO cells is 41%, 55%, and 18% for cultures in 2.00 µM, 1.00 µM, 0.50 µM Aβ, respectively. A single-factor analysis of variance (ANOVA) test, where appropriate, is used to identify statistical significance between experimental Aβ

concentration levels. Both AVOVA tests yield a significant ($p<0.05$) difference in cell concentration between trials. The difference in H441 cell concentration between trials is significant with a p-value of 3.34×10^{-8}. The difference in CHO cell concentration between trials is significant with a p-value of 1.36×10^{-6}. A post-hoc Tukey HSD test was run, and the Tukey HSD p-values as well as significance levels can be seen below in Table 2 A and B for H441 and CHO, respectively. For the H441 cells, there is a significant ($p<0.01$) difference between the 0.00 µM and 0.50 µM, 0.00 µM and 1.00 µM, and 0.00 µM and 2.00 µM. For the CHO cell trials, there is a significant ($p<0.01$) difference between the 0.00 µM and 1.00 µM, 0.00 µM and 2.00 µM, and 0.50 and 1.00 µM.

A control test was performed with TFA concentration 7.7 times greater than the concentration found in the experimental trials. Even at this concentration, it was found that there was not a significant difference ($p<0.05$) in cell proliferation rates between the TFA and the Aβ. The TFA did not significantly impact the change in cell concentration.

Table 2A. Concentration Treatment Pairs for H411 Cells

Treatments Pair	Tukey HSD p-value	Tukey HSD inference
[0.00] vs [0.50]	0.0010053	**p<0.01
[0.00] vs [1.00]	0.0010053	**p<0.01
[0.00] vs [2.00]	0.0010053	**p<0.01
[0.50] vs [1.00]	0.8999947	insignificant
[0.50] vs [2.00]	0.4903690	insignificant
[1.00] vs [2.00]	0.4199052	insignificant

Note. The Tukey HSD p-value and inference are shown above for treatment concentration pairs for H441 cells.. These parameters indicate a significant difference in cell concentrations for H441 cell control (0.00 µM) vs. 0.5 µM, 1.0 µM, and 2.0 µM Aβ.

Table 2B. Concentration Treatment Pairs for CHO Cells

[0.00] vs [0.50]	0.2612646	insignificant
[0.00] vs [1.00]	0.0010053	**p<0.01
[0.00] vs [2.00]	0.0010053	**p<0.01
[0.50] vs [1.00]	0.0026144	**p<0.01
[0.50] vs [2.00]	0.1235957	insignificant
[1.00] vs [2.00]	0.5010083	insignificant

Note. The Tukey HSD p-value and inference are shown above for CHO concentration of the treatment pairs. These parameters indicate a significant difference in cell concentrations for CHO cell control (0.00 µM) vs. 1.0 µM and 2.0 µM Aβ, and for 0.5 µM and 1.0 µM Aβ.

Discussion

The objective of this study was to determine the effects of Aβ on cell growth *in vitro* of H441 cells. The results indicated that Aβ is a potent inhibitor of cell proliferation in H441 cell cultures. This is supported by the ANOVA results which show, at a significance level of $p<0.05$, there is a statistically significant difference in cell growth (proliferation) when the H441 cells are exposed to Aβ at concentrations of 2.00 µM, 1.00 µM, and 0.50 µM. A control of 0.00 µM Aβ with an equivalent amount of solvent served as a positive control. The H441 and the CHO trials both show significant decrease in cell concentration. The results indicate Aβ demonstrates antiproliferative properties against H441 cells at a concentration greater than 0.50 µM.

It is common for mammalian cells to go through amyloidosis: the deposition of amyloid proteins in the body. Recent studies suggest periods of excessive environmental stress lead to an increase in the rate of amyloidosis. In other cancer types, such as breast and prostate, amyloidosis causes cells to enter a state of dormancy and decreased proliferation. Studies suggest that AD diagnoses lead to a decreased occurrence of breast cancer. Amyloid peptides are amphiphilic and can disrupt membrane dynamics. The aggregation of Aβ peptides near

the membranes may lead to an increased penetration of cell membranes, binding of receptors, and increasing the formation of endocytic vesicles. Prior studies with mammalian xenografts have shown the presence of amyloidosis leads to cancer cells entering a dormant, nonreplicating state. The state is augmented as the aggregation of amyloid fibers increase (Mizejewski, 2017).

In this experiment, the use of the vi-cell cell viability analyzer to measure the cell concentration was significantly more accurate than previous trials with a hemocytometer. While it was much more accurate, experimental errors resulted in the loss of eight H441 samples and 3 CHO samples out of the 192 collected. This resulted in a total sample size of 88 for the H441 cells and 93 for CHO cells.

Future studies will include experiments that seek to quantify whether the chelation of Aβ with certain metal ions could lead to a significant change in cell proliferation of cancer cells. Studies have indicated that Cu^{2+} has been shown to bind to Aβ and influence the interaction that the peptide has with cell membranes. This change in interaction may lead to the resting of a peptide on the bilayer surface rather than being integrated into the membrane (Lau et at., 2006). Additionally, the presence of Cu^{2+} and $Fe^{2+/3+}$ participate in the formation of neurotoxic reactive oxygen species (Shi et al., 2013). Conducting further experiments that test the ACP properties of Aβ when tested in conjunction with other anti-cancer treatments could yield results regarding the catalytic properties of AB. Certain limitations exist regarding the current knowledge of the mechanistic action of Aβ as an ACP, and future studies could provide clarification for the ACP properties of AB as part of the infection hypothesis.

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