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## **Amyloid-beta42 toxicity reduction in human neuroblastoma cells using cholera toxin b subunit-myelin basic protein expressed in chloroplasts**

Alexandra Ayache  
*University of Central Florida*



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AMYLOID-BETA42 TOXICITY REDUCTION IN HUMAN  
NEUROBLASTOMA CELLS USING CHOLERA TOXIN B SUBUNIT-  
MYELIN BASIC PROTEIN EXPRESSED IN CHLOROPLASTS

by

ALEXANDRA C. AYACHE

A thesis submitted in partial fulfillment of the requirements  
for the Honors in the Major Program in Molecular Biology and Microbiology  
in the College of Medicine  
and in The Burnett Honors College  
at the University of Central Florida  
Orlando, Florida

Summer Term 2012

Thesis Chair: Dr. Henry Daniell

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## ABSTRACT

Alzheimer's disease (AD) is an age progressive neurodegenerative brain disorder, affecting 37 million people worldwide. Cleavage of amyloid precursor protein by  $\beta$ - and  $\gamma$ -secretase produces the amyloid-beta ( $A\beta$ ) protein, which significantly contributes to AD pathogenesis. The  $A\beta$  aggregates, formed at the surface of neurons and intracellularly, cause neurotoxicity and decrease synaptic function. Inhibiting or degrading  $A\beta$  accumulation is a key goal for development of new AD treatments. Evidence shows that human Myelin Basic Protein (MBP) binds to and degrades  $A\beta$  thereby, preventing cytotoxicity. A potential method for oral drug delivery that will allow plant-derived bioencapsulated MBP to pass through intestinal epithelium and bypass denaturing stomach acidity is quite novel. Cholera Toxin B subunit (CTB), when fused with MBP, can serve as a vehicle for oral delivery of this chloroplast expressed therapeutic protein into the systemic circulation. Within chloroplast, CTB forms a pentameric structure that binds to GM1 ganglioside receptors, allowing receptor-mediated endocytosis. In order to investigate protein entry through neuronal GM1 receptors, we first created CTB fused to the green fluorescent protein (GFP). Incubation of this fusion protein with human neuroblastoma cells resulted in GFP entry into these cells whereas GFP alone was unable to enter. Similarly, co-incubation of CTB-MBP, via neuronal GM1 binding, allowed MBP to reduce neurotoxicity of  $A\beta_{42}$  treated cells by 37.1%. Delivery of CTB-MBP through GM1 receptor mediated binding should therefore facilitate oral administration, storage, heat stability and low cost AD treatment.

## **DEDICATIONS**

For my parents, who have supported me through all of my academic endeavors.

For my sister, who is my best friend.

## ACKNOWLEDGMENTS

This study was supported by NIH grants to Dr. Henry Daniell. I would like to express my gratitude to my Thesis Chair, Dr. Henry Daniell, for the opportunity to conduct research in his laboratory and for guiding me as a young scientist. I would like to thank Dr. Mohtashem Samsam, and Dr. James J. Hickman for serving on my committee and for sharing their experience and wisdom. I would like to thank Neha Kohli, who conducted the necessary preliminary protein quantification work and the preliminary GM1 ELISA binding assay. Neha also provided teamwork during the initial CTB-GFP experimentation. I thank Dr. Jogi Pattisapu for the generous gift of the human neuroblastoma cell line used in the experimentation for this thesis. I would like to express my gratitude to Dr. Kwangchul Kwon, who offered intellectual and experimental advice when needed. I would like to acknowledge Donevan Westerveld and Neha Kohli for the time they put into purifying the therapeutic protein used in this thesis. I would like to thank Dr. Meenu Madan and Dr. Yasser Saad for taking the time to look at some of my experimental data and giving their professional advice and thoughts. I would like to thank the other laboratory members of Dr. Daniell's lab including Dr. Dolendro Singh, Dr. Dheeraj Verma, Dr. Jin, Bindu, Aditya Kamesh, and Grace, who have been very helpful. I would also like to thank Denise Crisafi and Kelly Astro for their guidance throughout my Honors in the Major experience. Finally, I would like to thank God for giving me the will power to complete my Honors Thesis while I was taking a full course load, studying for the MCAT, and remaining involved in other extracurricular activities.

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## **LIST OF ACRONYMS/ABBREVIATIONS**

AD – Alzheimer’s Disease

APP – Amyloid Precursor Protein

A $\beta$  – Amyloid-Beta

MBP – Myelin Basic Protein

CTB – Cholera Toxin B subunit

GM1 – Ganglioside M1

GFP – Green Fluorescent Protein

LDH – Lactate Dehydrogenase

ELISA – Enzyme-linked immunosorbent assay

FBS – Fetal Bovine Serum

PBS – Phosphate-Buffered Saline

RCF – Relative Centrifugal Force

WT – Wild Type

## CHAPTER ONE: INTRODUCTION AND BACKGROUND

### Alzheimer's Disease and Pathogenesis

Alzheimer's disease (AD) is a neurodegenerative brain disorder, which becomes progressively worse with age and is eventually fatal. Over 37 million people worldwide suffer from AD. In 2010, AD had a global cost of over 600 billion dollars (1). Individuals who suffer from AD experience reduced cognitive function, including reductions in memory and intellectual activity. Currently there is no cure for AD. The medications prescribed for AD patients only help partially with symptoms not the cause. These medications target the enhancement of chemical messengers involved in memory, synaptic signaling and learning. Medications that stop the damage to brain cells have not yet been developed.

Cleavage of the amyloid precursor protein (APP) by  $\beta$ - and  $\gamma$ -secretase, produces the amyloid-beta ( $A\beta$ ) protein and excess accumulation of this protein in the brain is a significant contributor to the pathogenesis of AD (2, 3). Currently, the precise etiology of AD is inconclusive, but substantial evidence shows that  $A\beta$  plays a key role in the development of the disease (4-6). The proteolysis of APP produces  $A\beta$  peptides comprising of 39 to 43 amino acid residues. Among these  $A\beta$  peptides,  $A\beta_{42}$  significantly contributes to the initiation of  $A\beta$  aggregates, resulting in the formation of insoluble oligomers and eventually fibril structures (7, 8). The oligomeric structures and fibril aggregates of  $A\beta$  formed at neuronal membranes and synaptic endings predominantly cause neurotoxicity, decreased synaptic function and the progression of AD (9). Inhibiting and degrading  $A\beta$  aggregate formation have been considered

as key approaches for the development of new effective treatments for this neurodegenerative disease.

### **Myelin Basic Protein**

The human Myelin Basic Protein (MBP, molecular weight 18.5 kDa) has been shown to bind to A $\beta$  and prevent fibril formation (10). MBP is a major constituent of myelin sheaths of the central nervous system. The MBP purified from a bacterial recombinant expression system or from human brain effectively inhibits A $\beta$  aggregation (10), signifying that the activity is not dependent on post-translational modification. It was important to determine if post-translational modification is crucial for the inhibitory effects of MBP on A $\beta$  because MBP undergoes various post translational modifications in the brain. A recent study showed that the A $\beta$  binding site on MBP is within the first 64 residues of the N-terminal domain (10). Evidence shows that MBP can degrade A $\beta$  peptides and fibrillar formations (11). Moreover, MBP is able to inhibit cytotoxicity caused by A $\beta$  in a dose dependent manner (12). Previous studies have shown the ability of MBP to degrade neurotoxic A $\beta$  (11). However, a potential physiological method for oral drug delivery that will allow MBP to pass through the intestinal epithelium protected from acids and enzymes in the stomach is quite novel. In this study, we focus on developing an oral delivery method of MBP bioencapsulated in plant cells.

### **Oral Delivery of Chloroplast Expressed Therapeutic Proteins**

Currently, the cost of many prescription drugs and medical care are not affordable. Also, drug delivery and availability in developing countries is a major challenge because of cold chain

logistics as well as the need for trained individuals to administer the drug. Production of therapeutic proteins in plants enables oral delivery of biopharmaceutical proteins, mass production at a low cost, transportation without cold conditions, long-term storage, heat stability, and *in vitro* processing. Protection from the low pH of the stomach can be accomplished by bioencapsulation of the therapeutic protein in plant cells (13, 14). Cholera Toxin B subunit (CTB) (~11 kDa) has been used as a vehicle to get plant expressed therapeutic proteins into the systemic circulation via oral delivery (15). By creating a fusion protein of the therapeutic protein and CTB, the chimeric protein passes through the intestinal mucosal wall into the circulatory system (15, 16). This occurs because Ganglioside M1 (GM1) receptors on intestinal epithelial cells allow receptor-mediated endocytosis due to the binding ability of CTB to the GM1 receptor (16). Within chloroplast, CTB forms a pentameric structure, which binds to these GM1 receptors (16). In this study, we used CTB fused to MBP. A furin cleavage site along with a glycine proline glycine proline (GPGP) hinge region was engineered between CTB and MBP. The GPGP hinge prevents steric hindrance whereas furin cleavage facilitates release of the therapeutic proteins once they pass through intestinal epithelial cells (15, 17). Furin is a ubiquitous protease and it allows separation of the fusion proteins. One hundred percent cleavage is not guaranteed due to the turn over rate of furin. The quick turnover rate of intestinal epithelial cells and the recycling of GM1 receptors provide continual function (18, 19). Studies show that the blood-brain barrier has compromised integrity in Alzheimer disease patients (20, 21); therefore, this may allow therapeutic CTB-MBP to access the brain more easily. Also, GM1 receptors of the blood-brain barrier caveolae are a potential target for transportation of therapies to the brain (22, 23). For future studies, CTB-MBP fusion protein without a furin cleavage site will be beneficial

as it has more potential to reach GM1 receptors of the brain. The intestinal GM1 will allow CTB-MBP to enter the circulatory system and then travel to GM1 receptors of the brain allowing MBP greater access to A $\beta$  accumulated in neurons of the AD brain.

### **Ganglioside M1 of the Nervous System**

One of the functions of gangliosides is to act as cell surface receptors (24). Gangliosides are predominately found in the brain at synaptic endings and on membranes (25). Also, gangliosides are enriched in lipid raft domains, which are necessary for neuronal function and cell signaling (1). Lipid rafts play a role in APP amyloidogenic processing and A $\beta$  aggregation (26). Evidence shows that lipid raft disruption promotes A $\beta$  aggregation and that gangliosides, predominately GM1, in lipid rafts contribute to accelerating A $\beta$  plaque generation (27). GM1 is involved in the regulation of APP processing and is able to increase A $\beta$ 42 in a dose dependent manner (28). GM1 binds A $\beta$  and promotes further aggregation of A $\beta$  (1, 29). Lipid rafts are significantly abundant in neurons of the hippocampus of the brain, which is the brain's memory center. Lipid raft disturbance may contribute to the build up of toxic A $\beta$  oligomers on the hippocampal neurons of Alzheimer's disease patients (30). The binding of CTB-MBP to GM1 of these lipid rafts may prevent progressive A $\beta$  aggregation through a competitive binding mechanism.

Studies show that CTB can bind to GM1 on the surface of neurons and enter through endocytosis. Once CTB is taken into the neuron, it disperses in the cytoplasm, axoplasm and the dendrites (31, 32). In this study, we show that CTB facilitates entry of fused protein into neurons as well. We confirmed the presence of GM1 receptors on human neuronal cells by using plant-

derived CTB-Green Fluorescent Protein (GFP) fusion protein created via chloroplast genetic engineering. The presence of GM1 on neurons should facilitate receptor-mediated delivery of MBP to the site of A $\beta$  accumulation at neuronal cell membranes as well as to intracellular A $\beta$ . Therefore, co-incubation of CTB-MBP, via neuronal GM1 binding, should allow MBP to reduce the neurotoxicity of A $\beta$ 42 treated cells.

### **Intracellular Amyloid-beta, Phospho-Tau Protein and Future Applications**

A $\beta$  accumulation also occurs intracellularly. In addition to accumulation on the cell surface, studies show that A $\beta$  is generated in the golgi, endosomal/ lysosomal system and the endoplasmic reticulum (33, 34). Also, the mitochondria are sites of A $\beta$  accumulation; therefore, the functioning of the mitochondria of Alzheimer's Disease neurons is impaired (35). Due to the fact that brain cells require abundant energy, compromised mitochondria result in neurons losing their functioning abilities. Another pathological hallmark of AD is the phospho-Tau protein, which is contained in intracellular neurofibrillary tangles (36). Along with MBP targeting extracellular A $\beta$ , neuronal GM1 can potentially be used to get CTB-MBP into neurons for use in future experimentation that targets A $\beta$  that is generated inside neurons.

### **Lactate Dehydrogenase Cytotoxicity Assay**

The lactate dehydrogenase (LDH) cytotoxicity assay is used to assess cell death by measuring the amount of LDH released into the cell culture medium. When the cell membrane is compromised due to cell lysis or damage, the soluble cytosolic LDH enzyme is released from the cell. Therefore, the amount of LDH released into the cell culture medium is proportional to the

amount of cell death (37). The mechanism behind this assay consists of two reactions. First, LDH catalyzes the reaction in which lactate is oxidized to pyruvate and  $\text{NAD}^+$  is reduced, producing NADH and  $\text{H}^+$ . In the second reaction, diaphorase catalyzes the reduction of a tetrazolium salt by using the NADH and  $\text{H}^+$  produced in the first reaction. When the tetrazolium salt is reduced, it forms colored formazan. The absorbance of formazan can be detected at a wavelength of 490 nm. Therefore, the absorbance at 490 nm detected due to formazan is proportional to the amount of LDH released from the cells as a result of cytotoxicity.

### **Significance of the Research**

Research focusing on amyloid- $\beta$  aggregate inhibition, degradation and reducing its toxic effects using MBP on human neurons *in vitro* may significantly advance the development of novel therapeutics to treat Alzheimer's disease. This study shows that plant-derived CTB-MBP is functional in an *in vitro* assay; thereby, supporting parallel *in vivo* work. This research opens the door for a new way to treat Alzheimer's disease using plant-based CTB-MBP via oral delivery. Assessing the ability of human neurons to bind or uptake chloroplast derived CTB-GFP via GM1 receptors can serve as a model of the targeted delivery method for the CTB-MBP protein and its ability to reach the  $\text{A}\beta$  affected areas of neurons. Also, this study can lead to future research, which targets the destruction of intracellular  $\text{A}\beta$ .

### **Objectives**

The first aim of this study is to evaluate if CTB fused with GFP can bind to GM1 present on the neuronal cell surface and enter the cell, thus demonstrating the mechanism of how CTB



fused to therapeutic MBP could enter neurons and target intracellular A $\beta$ . The second aim of this study is to evaluate if plant-derived human MBP fused with CTB reduces the neurotoxic effects of amyloid-beta in order to develop an alternative and cost effective means to treat Alzheimer's disease.

### **Prior Work Performed in Dr. Daniell's Laboratory**

Transplastomic CTB-MBP tobacco plants were generated using gene gun delivery system. Tobacco leaves were bombarded with gold particles coated with chloroplast transformation vector containing CTB-hMBP fusion gene expression cassette. Using Southern blot analysis, the transformed plants were tested for transgene integration and homoplasmy. Further, immunoblot analysis confirmed transgene expression. Time and age specific leaves from transplastomic plants were harvested and immunoblotting using a CTB primary antibody was performed. Purification of CTB-MBP was challenging. The purity of purified protein was 33.1%. Therefore, the concentration of CTB-MBP is 33.1 ng/ $\mu$ l. Enrichment of CTB-MBP was accomplished by nickel column purification and was confirmed by western blots. Also, transplastomic CTB-GFP tobacco plants were generated. For quantification of CTB-GFP, densitometry was used. The concentration of crude CTB-GFP was 17.5 ng/ $\mu$ l. To confirm functionality, the ability of the chloroplast derived CTB-MBP fusion protein to bind to the GM1 receptor was established by a GM1 ELISA assay. Purified CTB and chloroplast derived CTB-MBP were compared and results showed that they have the same GM1 binding capability.

## **CHAPTER TWO: METHODS**

### **Cell Culture**

M17 human neuroblastoma cells were cultured in Opti-MEM® I Reduced Serum Media supplemented with 10% FBS, 1% Penicillin/Streptomycin in a 37°C incubator with full humidity and 5% CO<sub>2</sub>. For all protein incubation experiments, the treatment media consisted of only Opti-MEM® I reduced serum media (2% serum) excluding both FBS and Penicillin/Streptomycin. For experimentation, the neuroblastoma cells were plated in a 96 well plate at about 10<sup>4</sup> cells per well. Treatments were performed when cells were ~70% confluent.

### **CTB-GFP Extraction**

The leaf material was harvested from plants in the green house and observed under a UV lamp for significant GFP fluorescence. The substantially fluorescing leaves were selected and rinsed with water. The chosen leaves were ground using liquid nitrogen to make a fine powder. Three hundred microliters of cellgro® Phosphate-Buffered Saline, 1X (PBS) was added to 100 mg of finely ground plant leaf material. The mixture was then vortexed for 5 minutes at 4°C. After vortexing, the samples were sonicated for 5 seconds three times with a 30 second break in between. The material was then centrifuged at 4°C for 10 minutes. The supernatant was collected and recentrifuged for 10 minutes. The supernatant was collected again and filtered through a 0.45 and 0.2 micron filter consecutively to remove plant debris and to avoid contamination. The sample was kept on ice until used.

## **Determining the Ability of Plant Derived CTB-GFP to Bind to GM1 Receptors on Human Neuroblastoma Cells**

In order to investigate the presence of GM1 receptors on the neuronal surface of the M17 human neuroblastoma cell line and to determine if plant derived CTB fusion proteins could bind and enter the neuronal cell via the GM1 receptors, we used plant derived CTB-GFP. Extracted CTB-GFP and all control proteins were incubated with human neuroblastoma cells for 24 hours. The amount of CTB-GFP and control proteins added to each well was 350 ng. All treatments were done in triplicate. For negative controls, cells with no treatment, plant derived CTB-MBP, commercial GFP, Wild Type (WT) LAMD (low-nicotine tobacco) plant material, and commercial CTB were used. After the 24 hour incubation, cells were washed twice with PBS. Data was collected using microscopy. Cells in all wells were photographed using identical microscope settings (20X magnification and 4s GFP filter exposure). Data pictures were captured using a Nikon Eclipse TE2000-E microscope and the NIS ELEMENTS Advanced Research software.

## **Localization of CTB-GFP on Human Neuroblastoma Cells and Stage of Cell Cycle**

After confirming the presence of GM1 receptors on M17 Human Neuroblastoma cells and determining that CTB is essential for the fusion protein, GFP, to interact with the neuronal cell via GM1, it was important to determine where the GFP was localized upon interaction with the cell. In order to ensure very healthy cells that were less stressed due to excessive washings and aspirations, this follow up experiment was conducted using a 24 well plate. Cells were plated at  $\sim 10^6$  cells per well. M17 Human Neuroblastoma cells were incubated with 875ng

(23.3nM) CTB-GFP per well in triplicate. After the 24 hour incubation time point, cells were washed once with PBS. Data was collected using microscopy. Again, cells in all wells were photographed using a 20X magnification and a 4s GFP filter exposure. The same microscope stated above was used.

### **Preparation of Amyloid-beta42**

The Beta-Amyloid (1-42) peptide was purchased from AnaSpec. The peptide was reconstituted to the final concentration of 1mM using ddH<sub>2</sub>O and the pH was adjusted to 7. Before incubating A $\beta$ 42 with M17 human neuroblastoma cells, the A $\beta$ 42 was subject to pre-aggregation in 1X PBS for 16 hours at 37 °C. For the co-incubation of CTB-MBP and A $\beta$ 42, 100 mM NaCl was added to the pre-aggregate A $\beta$ 42 to increase aggregation. A $\beta$ 42 aggregation was confirmed by immunoblot using an anti A $\beta$ 42 antibody.

### **Cytotoxicity Caused by A $\beta$ 42 and Co-incubation of A $\beta$ 42 with CTB-MBP**

The concentration of A $\beta$ 42 that is optimally toxic to M17 Human Neuroblastoma cells was determined using a LDH cytotoxicity assay. Cytotoxicity was determined by measuring LDH release using the CytoTox96® Non-Radioactive Assay (Promega). Before treatment, growth media was aspirated from wells and replaced with 20  $\mu$ M or 40  $\mu$ M A $\beta$ 42 in treatment media. The experiment included cells in treatment media only as the negative control. Another set of cells in treatment media only contained cells for complete lysis and represented the maximum LDH release (100% cytotoxicity) control. All treatments were carried out in triplicates. After 48 hours, lysis buffer was added to the maximum LDH release control and left to incubate for 45

minutes. After confirmation of complete lysis by microscopy, 50  $\mu$ l from each well was transferred to a new 96 well plate and 50  $\mu$ l of reaction solution was added. The plate was incubated in the dark for 30 minutes and then stop solution (50  $\mu$ l) was added to each well. Absorbance values were determined at 490 nm using a plate reader. Percent cytotoxicity of control was calculated according to the equation:

$$\% \text{ Cytotoxicity} = \text{Experimental LDH release (OD490)} / \text{Maximum LDH release (OD490)}$$

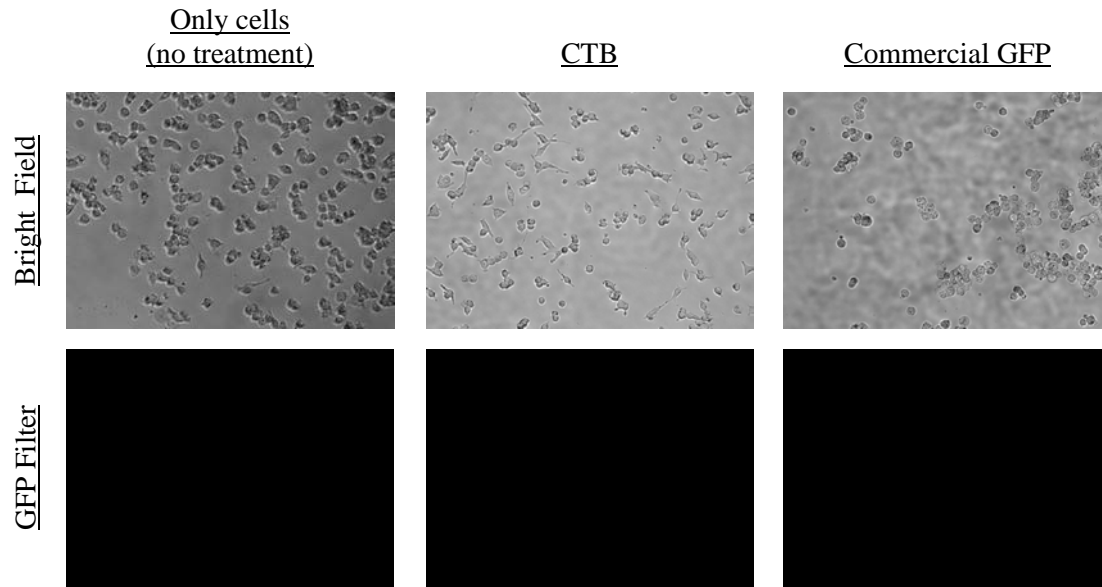
In order to assess the ability of CTB-MBP to reduce the cytotoxicity caused by A $\beta$ 42, a co-incubation experiment was performed. Cell death was assessed using the same LDH cytotoxicity assay. Before treatment, growth media was aspirated from the wells then replaced with 40  $\mu$ M A $\beta$ 42, 40  $\mu$ M A $\beta$ 42 plus 46.5 nM CTB-MBP, or 40  $\mu$ M A $\beta$ 42 plus 93 nM CTB-MBP in treatment media. The CTB-MBP protein was filtered through a 0.22 micron filter before use to avoid contamination. The same negative control and maximum LDH release control used in the cytotoxicity experiment were used for the co-incubation experiment. All treatments were done in triplicates. The relative cytotoxicity was determined by the assay described above. The only procedural difference in the co-incubation experiment was centrifugation of the plate at 200 RCF for 4 minutes before 50  $\mu$ l of supernatant from each well was transferred to a new 96 well plate. Centrifugation was performed in order to sediment any cellular debris in the culture media to enhance the accuracy of absorbance due to LDH.

## **CHAPTER THREE: RESULTS**

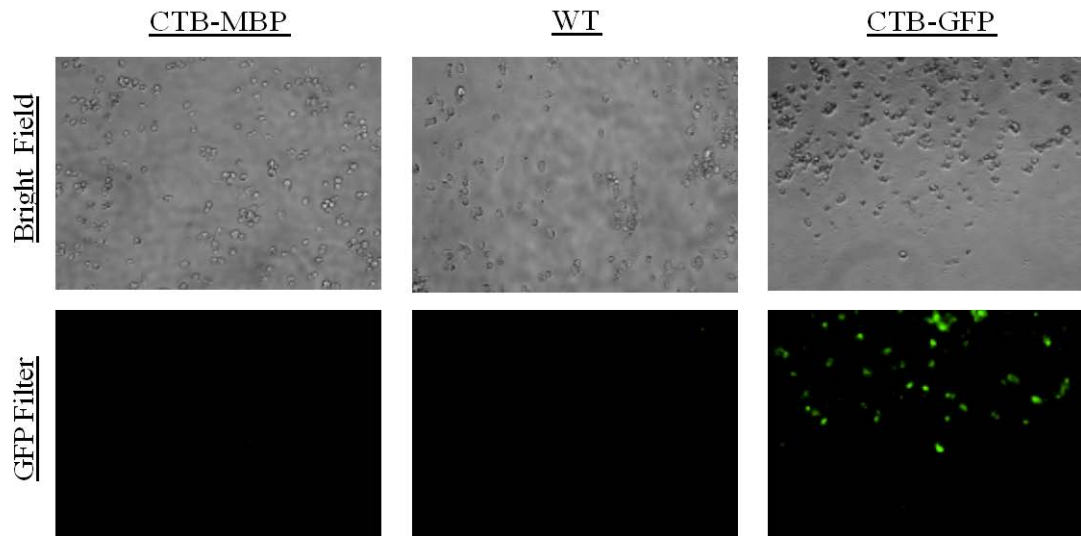
### **Plant Derived CTB-GFP Binds to GM1 Receptors on Human Neuroblastoma Cells**

To investigate the presence of GM1 receptors on M17 Human Neuroblastoma cells and to determine the ability of the CTB-GFP fusion protein to bind to neuronal GM1 receptors, cells were incubated with CTB-GFP or controls for 24 hours. GFP fluorescence was observed in human neuroblastoma cells after incubation with CTB-GFP (Figure 1). When the cells were incubated with commercial GFP alone or other controls under the same conditions, no GFP fluorescence was observed. These results show that CTB binding to neuronal GM1 is essential for uptake of GFP by human neuroblastoma cells.

**A**



**B**

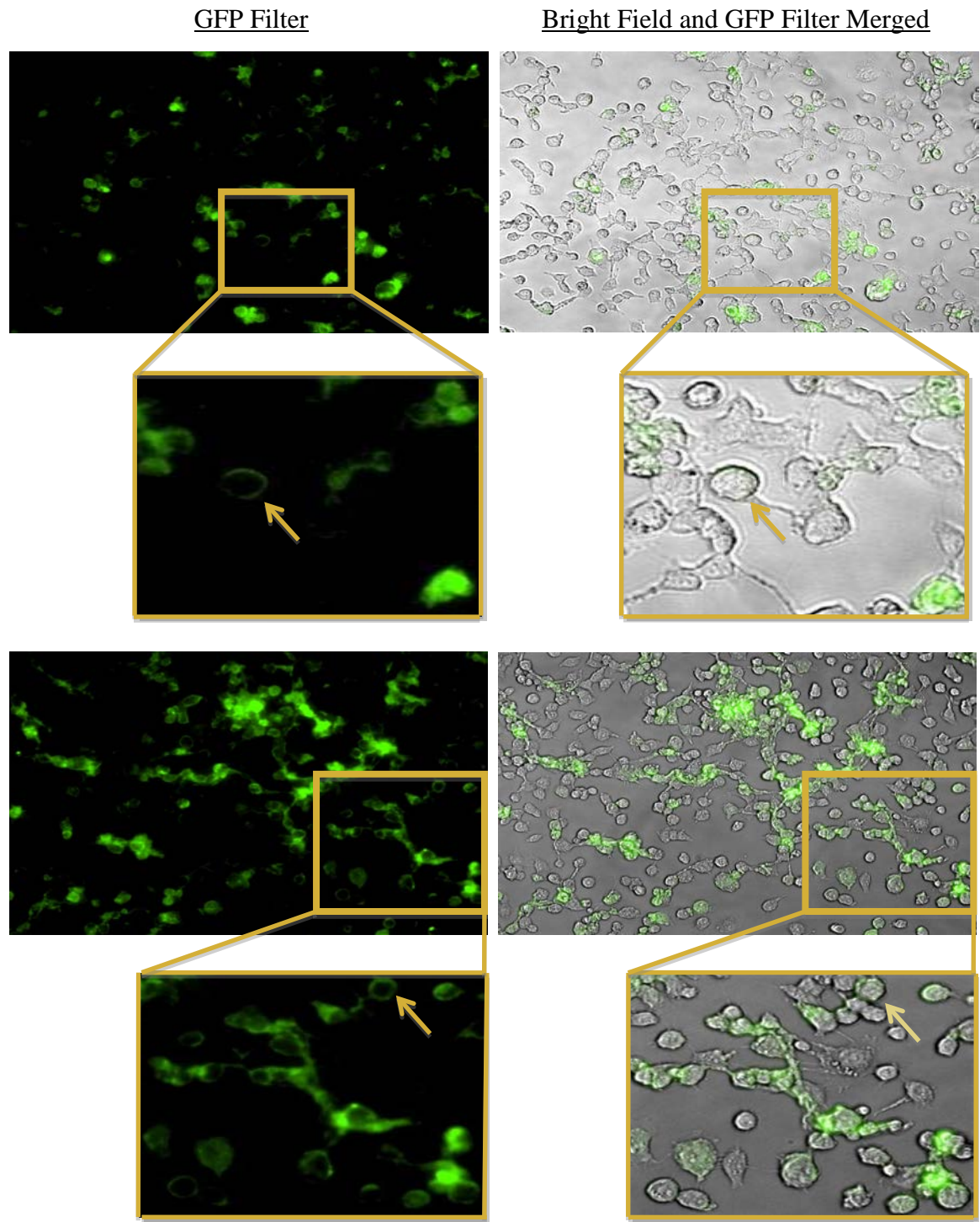


**Figure 1. Chloroplast Derived CTB-GFP Binds to GM1 Receptors on Human Neuroblastoma Cells.** GFP fluorescence from M17 Human Neuroblastoma cells was analyzed by fluorescence microscopy. Cells were incubated with equal amounts of controls or CTB-GFP. All pictures were taken at a magnification of 20X and the GFP filter was set for a 4s exposure for all samples. Panel A shows commercial controls including GFP alone. Panel B shows plant derived controls and CTB-GFP.

### **Localization of CTB-GFP on Human Neuroblastoma Cells and Stage of Cell Cycle**

After confirming the presence of GM1 receptors on M17 Human Neuroblastoma Cells and determining that CTB is essential as a carrier to deliver GFP into the neuronal cell via GM1, location of GFP fluorescence was examined. Neuroblastoma cells were incubated with CTB-GFP for 24 hours under less harsh conditions to ensure that the cells bound CTB-GFP without being stressed or having a compromised membrane. GFP fluorescence was observed on the cell surface or intracellularly depending on the stage of the cell's life cycle (Figure 2). These results show that through binding to the neuronal GM1 receptor, human neuroblastoma cells can uptake the CTB-GFP fusion protein.

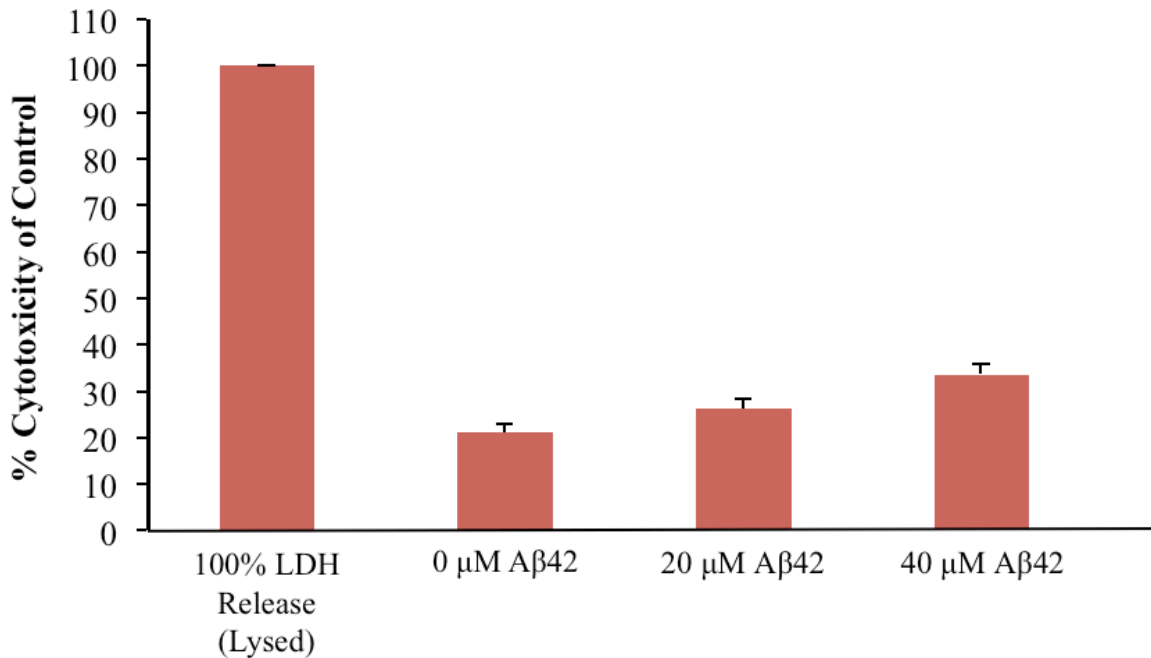




**Figure 2. Localization of CTB-GFP on Human Neuroblastoma Cells and Stage of Cell Cycle.** M17 Human Neuroblastoma cells were incubated with chloroplast derived CTB-GFP for 24 hours. Pictures were taken at a magnification of 20X under the GFP filter for 4s. The boxes show enlarged images of the indicated region and the location of GFP fluorescence on the surface or within neurons. Arrows indicate CTB-GFP binding on the neuron surface. Some cells show internalized GFP.

### Cytotoxicity Caused by A $\beta$ 42

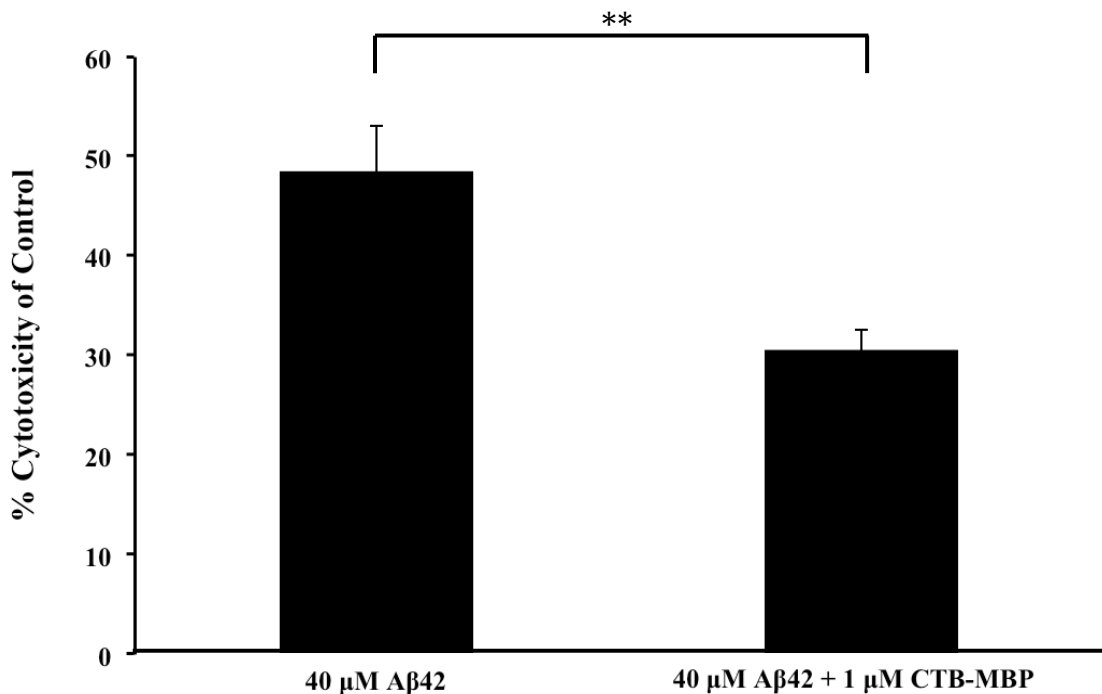
In order to determine the optimal concentration of Amyloid-beta42 that has toxic effects on M17 Human Neuroblastoma cells, cells were incubated with different concentrations of A $\beta$ 42 for 48 hours (Figure 3). A $\beta$ 42 was pre-aggregated for 16 hours at 37 °C before treatment of cells. A concentration of 40  $\mu$ M A $\beta$ 42 and an incubation time of 48 hours were selected as appropriate experimental conditions for the further co-incubation experiments with CTB-MBP. Further investigation was done before the co-incubation experiment, as a 50% cell death mark was not reached. It was determined that 100 mM NaCl should be added to the pre-aggregate A $\beta$ 42 material to enhance aggregate formation before treatment with cells.



**Figure 3. Cytotoxicity of Human Neuroblastoma Cells Caused by A $\beta$ 42.** Percent cytotoxicity was determined by comparison to a 100% LDH release (lysed) control, which had an average absorbance of 0.835. Data are shown as mean  $\pm$  S.D. of values obtained from triplicate experiments.

### Reduction of Neurotoxicity Caused by A $\beta$ 42 via Co-incubation with CTB-MBP

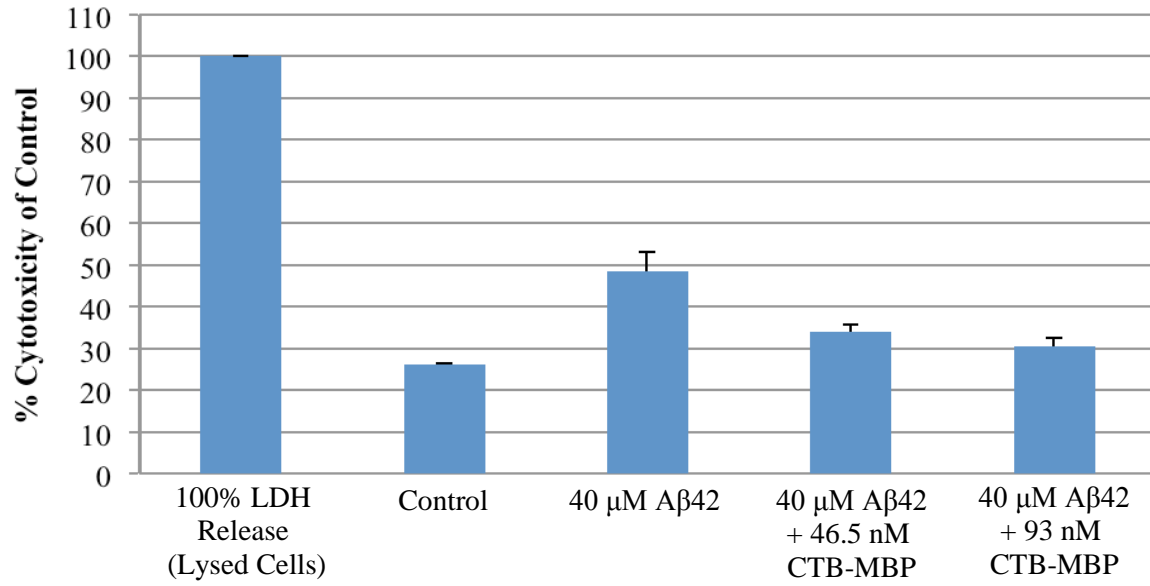
The ability of plant-derived CTB-MBP to reduce cytotoxicity caused by A $\beta$ 42 was assessed by co-incubation of A $\beta$ 42 and CTB-MBP with human Neuroblastoma cells. Plant derived CTB-MBP was co-incubated with the previously determined optimal toxic concentration and conditions of A $\beta$ 42 for 48 hours. As shown in Figure 4, the cytotoxic effect of A $\beta$ 42 was reduced by 37.1% when CTB-MBP was co-incubated with A $\beta$ 42 treated human neuroblastoma cells.



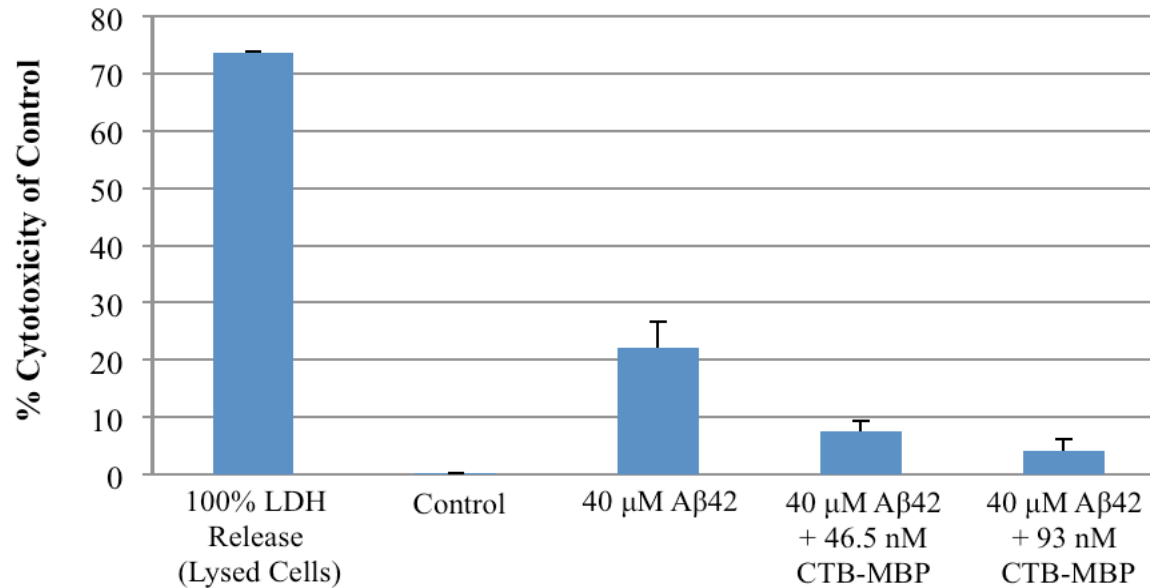
**Figure 4. Chloroplast Expressed CTB-MBP Reduces the Neurotoxic effect of A $\beta$ 42.** M17 Human Neuroblastoma cells were incubated with 40 $\mu$ M A $\beta$ 42 with or without 1  $\mu$ M CTB-MBP for 48 hours. Percent cytotoxicity was determined by comparison to a 100% LDH release (lysed) control, which had an average absorbance of 0.588. Data are shown as mean  $\pm$  S.D. of values obtained from triplicate experiments. \*\*,  $p < 0.01$ . The statistically significant difference between the groups was calculated using single factor ANOVA.

## Complete CTB-MBP and A $\beta$ 42 Co-incubation Experiment

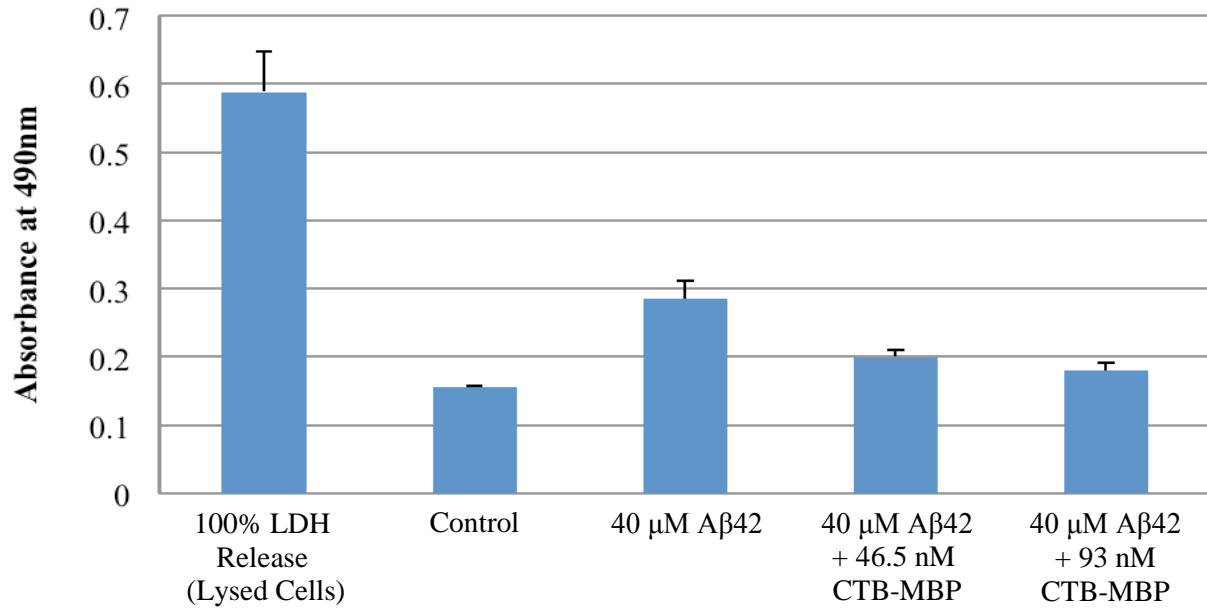
A



B



C



**Figure 5. Chloroplast Expressed CTB-MBP Reduces the Neurotoxic effect of Aβ42.** M17 Human Neuroblastoma cells were co-incubated with two different concentrations of CTB-MBP protein and cytotoxic concentration of Aβ42. Percent cytotoxicity was determined by comparison to a 100% LDH release (lysed) control, which had an average absorbance of 0.588. Data are shown as mean ± S.D. of values obtained from triplicate experiments. **A.** Cytotoxicity represented as percent of control. **B.** Cytotoxicity represented as percent of control and the negative control (only cells with treatment media, no Aβ42 or CTB-MBP) subtracted from all values. **C.** Cytotoxicity represented as absorbance at 490nm.

## CHAPTER FOUR: DISCUSSION

Ligand–receptor interaction on the cell surface is an essential mechanism to allow protein entry into cells. In this study, we showed the binding activity of CTB to GM1 receptors present on M17 human neuroblastoma cells and the invagination of a CTB fusion protein into the neuronal cell using fluorescent microscopy analysis. GM1 is known to serve as a receptor for pentameric CTB and has been extensively studied in regard to its interaction on intestinal epithelial cells (16). However, investigations concerning CTB fusion protein entry into brain cells are limited. The targeted delivery of therapeutic proteins into neurons via receptor-ligand (GM1-CTB) interaction should facilitate development of novel Alzheimer’s disease treatments, which target the intracellular pathological hallmarks of the disease, amyloid-beta and phospho-tau. Also, this concept can be utilized in treating other neurological diseases, such as Parkinson’s and Huntington’s disease.

To investigate the interaction of CTB fused protein with neuronal GM1, we used the M17 human neuroblastoma cell line. Strong GFP fluorescence was observed after the incubation of CTB-GFP and the cells when compared to none after incubation with GFP alone. The GFP fluorescent signals were detected around cell membrane and intracellularly demonstrating that CTB-GFP can enter the cell after the binding of CTB to GM1. Hence, CTB is necessary for GFP entry into neuronal cells via CTB-GM1 interaction. Fluorescence microscopy data shows that the location of GFP in human neuroblastoma cells depends on the cell cycle stage. The binding ability of CTB fusion proteins to neuronal GM1 on the cell surface and uptake of the fusion

proteins into the cell can serve as a targeted delivery route for treatments against Alzheimer's disease.

MBP has been shown to degrade and inhibit A $\beta$  assembly (10, 11). Thereby, CTB-MBP could be used as a therapy to reduce the neurotoxicity caused by A $\beta$  and inhibit the progression of neurodegeneration. Once MBP enters the neuron, via the mechanism of CTB binding to GM1, it can reach intracellular A $\beta$  and prevent aggregate formation as well as degrade the toxic accumulations.

GM1 of the brain should allow CTB to direct therapeutic MBP to extracellular A $\beta$  plaques, which inhibit synaptic function and contribute to neuron death. MBP can then protect neurons from damage by degrading A $\beta$  formations as well as inhibiting further aggregation. In this study, neurotoxicity caused by A $\beta$ 42 was simulated *in vitro*. We demonstrated that A $\beta$ 42 is toxic to human neuroblastoma cells and that neurotoxicity occurs in a concentration dependent manner. After optimization, it was established that pre-aggregated 40  $\mu$ M A $\beta$ 42 can cause significant human neuroblastoma cytotoxicity. The cell death caused by A $\beta$ 42 was inhibited using plant derived CTB-MBP. These results are consistent with a previous study that showed MBP caused increased cell viability of A $\beta$ 42 treated rat cortical neurons (7). Co-incubation of the determined toxic concentration and conditions of pre-aggregated A $\beta$ 42 and about 1  $\mu$ M CTB-MBP resulted in a 37.1% decrease in human neuroblastoma cell death. Also, increasing concentrations of CTB-MBP resulted in increasing reductions of cell death. These results indicate that chloroplast expressed CTB-MBP is able to reduce the neurotoxic effects of aggregated A $\beta$ 42.

Reducing and preventing neuron damage caused by amyloid-beta aggregates can lead to potential treatments that hinder the progressive nature of the neurodegenerative Alzheimer's disease process. Through chloroplast genetic engineering techniques, CTB-MBP can be expressed at higher levels in plants leading to large-scale production at a lower cost. Bioencapsulation of the plant derived CTB-MBP may lead to a clinical treatment for Alzheimer's disease that can be delivered orally.



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