

# Contrasting the Effects of Alteplase and DAPT on the Cleavage of C-Terminal Fragments and Amyloid- $\beta$ Concentration

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### ABSTRACT

Alzheimer's disease is a very common type of dementia that triggers memory loss and impedes other important mental functions [1,2]. It is a neurodegenerative disorder which affects over 5 million people in the United States alone [1-3]. In fact, it is the 6th leading cause of death in the United States, and on average, an American is diagnosed with Alzheimer's disease every 66 seconds [1,3]. This can be a familial or sporadic disease which is primarily caused by the destruction of neurons which starts from the hippocampus and spreads throughout the brain (cerebellum is spared) [4]. The apoptosis of the countless neurons seems to be caused by a multitude of factors including amyloid-beta plaques, Tau tangles, and neuronal loss [1,2]. For the purposes of this investigation, there will be a primary focus on the amyloid-beta plaques because the buildup of neurotoxic plaques on the neurons seems to be a key factor in Alzheimer's disease [1,2]. Enzymes called  $\gamma$ -secretase and  $\beta$ -secretase cleave a protein called an amyloid precursor protein (APP) to form these amyloid-beta peptides which can accumulate and form neurotoxic plaques [5,6]. Previous studies have found that DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), a dipeptide analogue, is effective in inhibiting  $\gamma$ -secretase thus decreasing amyloid-beta concentration in the brain [6]. This study confirms the efficacy of DAPT in inhibiting  $\gamma$ -secretase, but also investigates the alternative inhibitory effects of drugs like Activase® rt-PA (alteplase), a tissue plasminogen activator typically used for treatment of stroke, and clonazepam (E64), a pill used to treat panic disorder and anxiety. Although the goal of this research was to observe the effects on both A $\beta$ 40 (40 amino acid amyloid-beta chain) and A $\beta$ 42 (42 amino acid amyloid-beta chain) production, only the effects of A $\beta$ 40 production were examined due to possible contamination in the A $\beta$ 42 tests.

### Keywords

DAPT, Alteplase, Clonazepam, Amyloid  $\beta$ -peptide, Alzheimer's disease therapeutics,  $\gamma$ -secretase, Protease inhibitors, Neuroblastoma cells, Amyloid precursor protein (APP).

### Formation of Amyloid Plaques in Alzheimer's disease

These plaques are commonly referred to as amyloid plaques because they are comprised of amyloid-beta polypeptides which denote a specific set of amino acids on a protein called amyloid precursor protein (APP) [1,7]. The true function of APP and amyloid-beta is unknown to the scientific community but is believed to play a critical role in neural growth [7,8]. APP is broken down or cleaved by enzymes called secretases of three types:  $\alpha$ ,  $\beta$ ,  $\gamma$ -secretase [5,6,9].

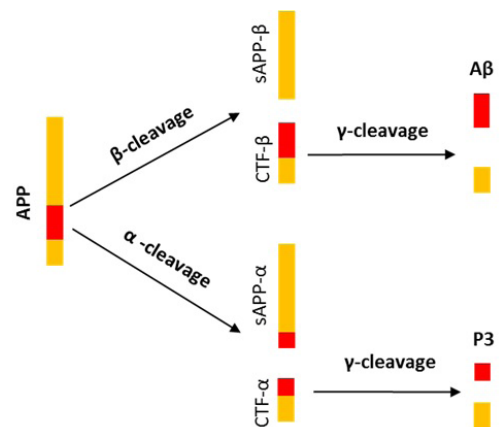


Figure 1:  $\alpha$ ,  $\beta$ ,  $\gamma$ -cleavage processes of APP.

As denoted in Figure 1, the red portion of the orange APP represents the amyloid-beta. Through  $\alpha$ -cleavage (using  $\alpha$ -secretase), the amyloid-beta is cleaved into two separate pieces, and this prevents the accumulation of amyloid plaques because the complete amyloid-beta polypeptide is not separated from the APP through  $\gamma$ -cleavage (using  $\gamma$ -secretase) [9]. Conversely, through  $\beta$ -cleavage (using  $\beta$ -secretase), the amyloid-beta polypeptide retains its full size when the APP is cleaved [5]. Through  $\gamma$ -cleavage, the amyloid-beta is distinctly separated from the rest of the APP as shown in Figure 1 [5,6]. According to previous studies, amyloid-beta peptides tend to have a half-life of approximately thirty minutes where it performs its function and then is degraded by an enzyme called endothelin-converting enzyme (ECE) [10-12]. The formation of this polypeptide may be beneficial in moderation, however, with great accumulation of amyloid-beta, the formed plaques will become toxic for the neurons thus resulting in the cell's death [8,12]. This is believed to be one of the main causes of memory loss and malfunction of tissues which prevents the nervous system from performing vital mental functions causing Alzheimer's disease [1,2].

### Objective

The objective of this study is to analyze the effects of DAPT, Activase® rt-PA, and clonazepam on A $\beta$ 40 and A $\beta$ 42 concentrations. The designed experiment investigates the combinatorial effects of these drugs on A $\beta$  levels. The study also examines the effects of these drugs on the concentration of C-terminal fragments. Based on the results, the study draws conclusion on the inhibitory effects of these drugs on APP processing enzymes. Western Blotting and ELISA tests are predominantly used to examine these effects.

### Methods and Materials

#### Cell Culture

Neuroblastoma cells were used as these cells have similar characteristics to neurons, and they have an immortal cell line since they divide rapidly. These cells have been specifically transfected to overexpress APP. A flask containing human neuroblastoma cells and media was withdrawn from an incubator kept at 37°C. The phenyl red served as a pH indicator in the media to observe any sign of contamination, but this was not evident. Since the cells were at a state of confluency where a monolayer of cells had formed and adhered to the bottom of the flask, it was ensured that the cells become suspended in the media. To accomplish this, the media was separated, and the cells were washed with 10 mL of phosphate buffered saline (PBS). After removing the PBS, 2 mL of trypsin, an enzyme that detaches the cells from the bottom of the flask, was added. Before the experiment, the trypsin was kept at approximately 37°C as the ideal temperature for the use of trypsin is body temperature. Cells were allowed approximately five minutes to become suspended in the solution. Immediately after five minutes, 8 mL of media was added to dilute the trypsin solution. With the aid of a hemocytometer, it was calculated that there were on average, 60 cells per quadrant. Finally, media was added back to the wells so that the concentration of the cells were calculated to be  $1.2 \times 10^6$  cells/mL. Cells and media were then distributed to two 12-well plates (4x3) as shown in Figures 2 and 3.

After overnight incubation, drug samples were added to the well plates. For the sake of convenience, the Activase® rt-PA (alteplase), DAPT, and clonazepam (E 64) is referred to as Drug 1, Drug 2, and Drug 3 respectively for the rest of the experiment. The 3 rows of the first plate (plate A) held the control, Drug 1, and Drug 2 respectively. In the second plate (plate B), the three rows had samples of Drug 3, Drug 1 & 2, and Drugs 1 & 3 respectively. Combination Drugs 2 & 3 or a combination of all three drugs were not tested and were not part of the scope of this research. The following tables (Tables 1 and 2) illustrate the volumes and concentrations of each drug needed for the experiment:

	Stock Concentration	Final/Desired Concentration
Drug 1	10 mM	100 $\mu$ M
Drug 2	10 mM	10 $\mu$ M
Drug 3	10 mM	100 $\mu$ M

**Table 1:** Desired concentration of each drug.

Using the formula:  $C_1 V_1 = C_2 V_2$

$C_1$  = Stock Concentration of drug

$C_2$  = Final/Desired Concentration of drug

$V_2$  = Final/Desired Volume of well

To calculate  $V_2$ , 0.5 mL/well x 4 wells/row = 2 mL/row

Drug 1:  $10,000 \mu\text{M} \times V_1 = 100 \mu\text{M} \times 2 \text{ mL}$   
 $V_1 = 0.02 \text{ mL} = 20 \mu\text{M}$  of Drug 1

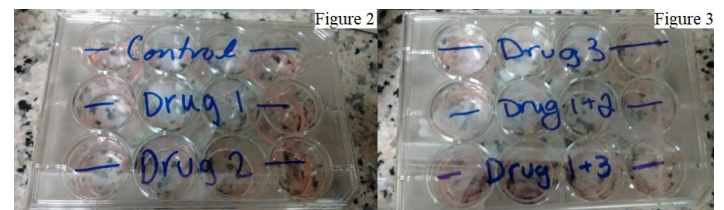
Drug 2:  $10,000 \mu\text{M} \times V_1 = 10 \mu\text{M} \times 2 \text{ mL}$   
 $V_1 = 0.002 \text{ mL} = 2 \mu\text{M}$  of Drug 1

Drug 3:  $10,000 \mu\text{M} \times V_1 = 100 \mu\text{M} \times 2 \text{ mL}$   
 $V_1 = 0.02 \text{ mL} = 20 \mu\text{M}$  of Drug 1

Plate and Row	Drug	Volume of Drug	Volume of Media	Total Volume
Plate A, Row 1	Control	0 mL	2 mL	2 mL
Plate A, Row 2	Drug 1	0.02 mL	1.98 mL	2 mL
Plate A, Row 3	Drug 2	0.002 mL	1.998 mL	2 mL
Plate B, Row 1	Drug 3	0.02 mL	1.98 mL	2 mL
Plate B, Row 2	Drugs 1 & 2	$0.02 + 0.002 = 0.022 \text{ mL}$	1.978 mL	2 mL
Plate B, Row 3	Drugs 1 & 3	$0.02 + 0.02 = 0.04 \text{ mL}$	1.96 mL	2 mL

**Table 2:** Desired volume of each drug.

After adding the specified volumes of drug and media to each well in each row, Plates A & B (Figures 2 and 3) were incubated overnight.

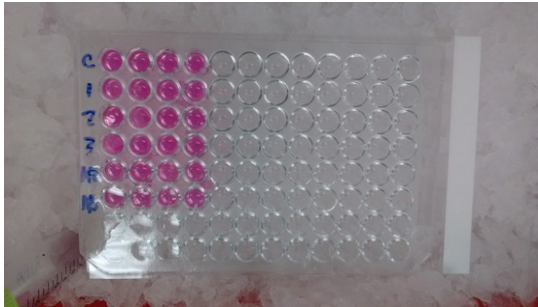


**Figure 2:** Plate A; **Figure 3:** Plate B.

#### Preparation of Primary Antibody

At this stage of the experiment, enzyme-linked immunosorbent

assay (ELISA) tests were conducted to calculate the drugs' effects on A $\beta$ 40 and A $\beta$ 42 production. Two 96-well plates (one for amyloid-beta 40 and one for amyloid-beta 42) were used for the assay. Based on a molar balance, 118  $\mu$ L and 141  $\mu$ L of primary mouse capturing antibodies were added to each well for A $\beta$ 40 and A $\beta$ 42, respectively. After five minutes, all the wells were washed with 300  $\mu$ L of PBS, and subsequently, 300  $\mu$ L of blocking agent (Block ACE) was added to each well to block all nonspecific binding sites.



**Figure 4:** 96-well Media plate.

### Intracellular and Extracellular Protein Samples

After preparing the ELISA plates, cells were removed from incubation to ensure that the cells were 100% confluent. Both the extracellular and intracellular proteins were taken into account to detect and capture all amyloid-beta proteins. Two hundred (200)  $\mu$ L of media was removed from each of the wells and distributed into a separate 96-well plate as shown in Figure 4 to collect the extracellular proteins. The 300  $\mu$ L of media left in each well was

discarded. Before attempting to collect the intracellular proteins, 500  $\mu$ L of PBS was added to each well to wash the cells. A pipette was used to scrape and detach cells from the walls and bottom of each well. The suspended cells from each well were collected and transferred to respective microcentrifuge tubes. The washing and scraping of cells was repeated so that each microcentrifuge tube had 1 mL of cell solution. There were four microcentrifuge tubes for the control, Drug 1, Drug 2, Drug 3, Drug 1 & 2, and Drug 1 & 3 for a total of 24 tubes. This entire process was conducted on ice at 4°C as a low temperature helps to slow the cell's metabolism down and hinder any enzymes from degrading the proteins. Samples were centrifuged for about 10 minutes. An aspirator was utilized to withdraw PBS from each tube, but the cell pellet at the bottom of the tube was left undisturbed. Subsequently, 100  $\mu$ L of radioimmunoprecipitation assay buffer (RIPA buffer) was added to each tube to lyse the cells.

With three of the microcentrifuge tubes of each sample, 100  $\mu$ L of lysed cells for each sample were added to wells in columns 4, 5, and 6. With the 96-well plate with media (Figure 4), 50  $\mu$ L of media for each sample was added to columns 7, 8, and 9 along with 50  $\mu$ L of EC buffer for sample dilution and coating. The standard curve (columns 2 and 3) was also created with 100  $\mu$ L of EC buffer with concentrations of 3.125 fmol/mL, 12.5 fmol/mL, 50 fmol/mL, 200 fmol/mL, and 800 fmol/mL. Table 3 represents the ELISA plate.

After completing this, the two ELISA plates for each protein were incubated for 24 hours where three tubes of each sample were used.

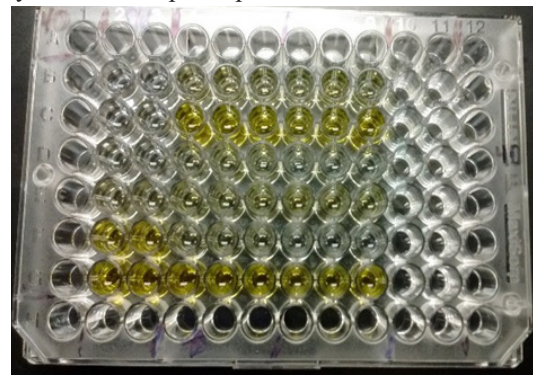
	1	2	3	4	5	6	7	8	9	10	11	12
A		<b>Standard</b>		<b>Intracellular Proteins</b>			<b>Extracellular Proteins</b>					
B	Control	100 $\mu$ L of EC buffer		100 $\mu$ L of Control Samples			50 $\mu$ L of EC buffer and 50 $\mu$ L of Control Sample's Media					
C	Drug 1	100 $\mu$ L with 3.125 fmol/mL conc.		100 $\mu$ L of Drug 1 Samples			50 $\mu$ L of EC buffer and 50 $\mu$ L of Drug 1 Sample's Media					
D	Drug 2	100 $\mu$ L with 12.5 fmol/mL conc.		100 $\mu$ L of Drug 2 Samples			50 $\mu$ L of EC buffer and 50 $\mu$ L of Drug 2 Sample's Media					
E	Drug 3	100 $\mu$ L with 50 fmol/mL conc.		100 $\mu$ L of Drug 3 Samples			50 $\mu$ L of EC buffer and 50 $\mu$ L of Drug 3 Sample's Media					
F	Drug 1 & 2	100 $\mu$ L with 200 fmol/mL conc.		100 $\mu$ L of Drug 1 & 2 Samples			50 $\mu$ L of EC buffer and 50 $\mu$ L of Drug 1 & 2 Sample's Media					
G	Drug 1 & 3	100 $\mu$ L with 800 fmol/mL conc.		100 $\mu$ L of Drug 1 & 3 Samples			50 $\mu$ L of EC buffer and 50 $\mu$ L of Drug 1 & 3 Sample's Media					
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**Table 3:** Descriptions of contents of the wells of the 96-well ELISA plate.

### Final Addition of Substrates

To complete the ELISA plates, secondary antibodies were added to each well after removing the plates from incubation. First, each well was washed with 300  $\mu$ L of PBS three times. For the A $\beta$ 40 plate, diluted 1:4000 A $\beta$ 40 secondary antibody was applied. For the A $\beta$ 42 plate, diluted 1:1000 A $\beta$ 42 secondary antibody was added. 100  $\mu$ L of the respective solutions were added to each well in each plate and the ELISA plates were allowed to incubate for 4 hours. After 4 hours, a combination of TMB (3,3',5,5' - tetramethylbenzidine) substrate and horseradish peroxidase was added to the wells of both plates. After waiting about 15-20 minutes, blue color changes in the wells were evident in the A $\beta$ 40 plate, however, minimal color change was observed in the A $\beta$ 42 plates. From this experiment, it was believed that the A $\beta$ 42 plate must have been contaminated. Because of this, the A $\beta$ 42 plate was disregarded. 100  $\mu$ L of stop solution (sulfuric acid) was added to

each well of the A $\beta$ 40 plate to complete the assay, and the contents of the wells became yellow as shown in Figure 5. The plates were then analyzed under a spectrophotometer.



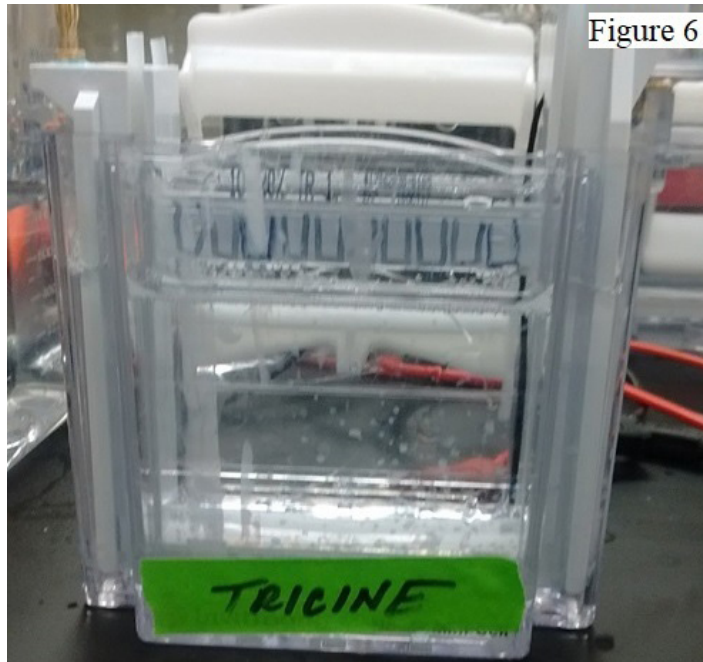
**Figure 5:** A $\beta$ 40 ELISA Plate.

## Gel Electrophoresis

The fourth microcentrifuge tube for each mixture was used for a gel electrophoresis experiment. Tricine loading buffer and beta mercaptoethanol was added to each of the remaining tubes. The tubes were sterilized at 95°C, and 15 µL of each sample was added to the gel to perform gel electrophoresis. In Table 4, each box represents a well in the gel.

Ladder	Control	D1	D2	D3	D1 & 2	D1 & 3	Control	D1 & 2	D1 & 3
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**Table 4:** Order of samples added to the wells of the gel for gel electrophoresis.



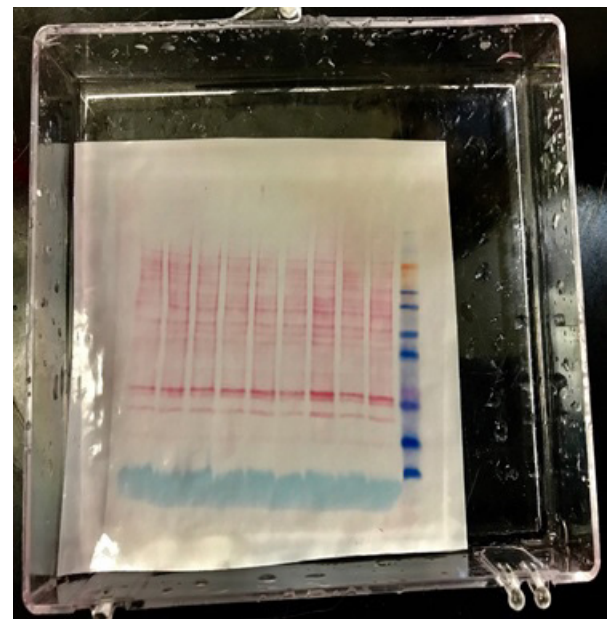
**Figure 6**

**Figure 6:** Gel Electrophoresis running chamber.

After placing the lid on the running chamber (Figure 6), the electrodes on the lid were connected to a voltmeter set at 120V. After one hour, the gel was removed and placed in a cassette. In the cassette, a membrane was placed next to the gel for the proteins to transfer onto. A sponge and a filter paper were added on either side of the gel with the membrane to protect the gel and to ensure that the proteins blot onto the membrane. The cassette was attached to the transfer chamber at 100V. After one hour, the proteins on the membrane were stained with Ponceau S (red) as shown in Figure 7.

## Western Blotting

After staining the proteins, Bovine Serum Albumin (BSA) was used to block nonspecific sites. The membrane was washed with PBS to prepare for the addition of primary antibodies. With the addition of primary antibodies, it was incubated overnight. The membrane was washed three times with PBS. The secondary antibodies were added, and the membrane was placed on a Belly Dancer™ platform shaker for an hour. Washing with PBS was repeated. The results of the Western Blots were viewable through a luminescent image analyzer after thirty minutes.

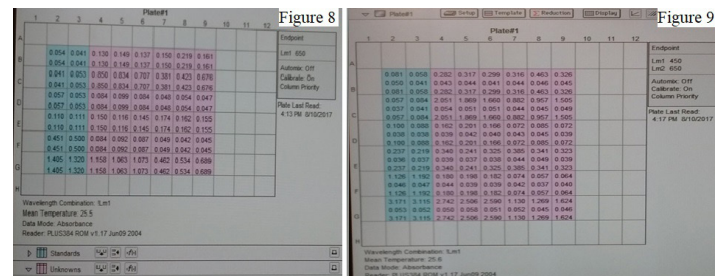


**Figure 7:** Membrane showing Gel Electrophoresis results.

## Results and Analysis

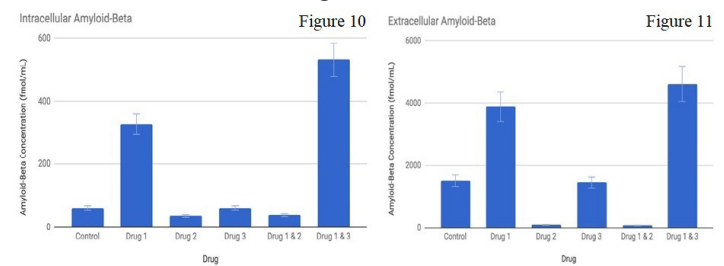
### ELISA Results

ELISA plates were analyzed using a SPECTRA max PLUS 384 spectrophotometer. The optical density was read at 2 wavelengths, 450 nm, and 650 nm. The absorbance values of each well are shown in Figure 8 and 9 for before and after the addition of stop solution.



**Figure 8:** Absorbance values Before Stop Solution. **Figure 9:** Absorbance values After Stop Solution.

The spectrophotometer readings of absorbance values were used to determine intracellular and extracellular amyloid-beta concentration as shown in Figures 10 and 11.



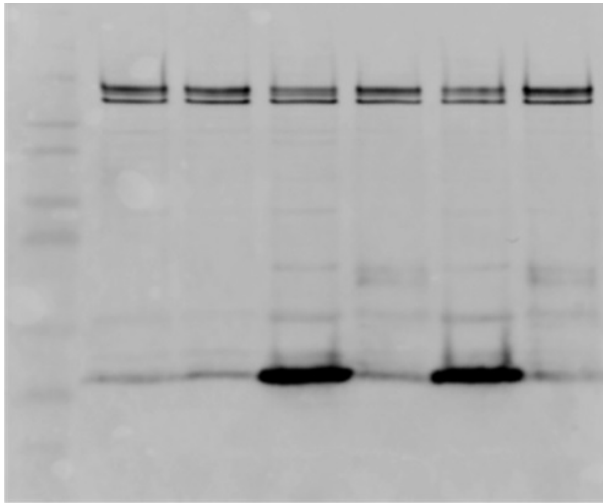
**Figure 10:** Intracellular Amyloid-Beta Concentrations. **Figure 11:** Extracellular Amyloid-Beta Concentrations.

From the data presented in Figure 10 and Figure 11, it can be

observed that Drug 1 significantly increases intracellular and extracellular A $\beta$  production, whereas Drug 2 decreases A $\beta$  production, especially extracellular A $\beta$ . However, Drug 3 seems to have no change at all to intracellular or extracellular A $\beta$  production as the value for Drug 3 and the Control are almost identical in both graphs. When Drugs 1 & 2 are combined, overall A $\beta$  production seems to decrease similarly to Drug 2 alone. On the other hand, when Drug 3 is combined with Drug 1, the increase in A $\beta$  concentration is even greater than the results of Drug 1 only.

### Western Blot Results

The membrane for Western blot was analyzed using a GE ImageQuant LAS 4000 mini Luminescent Image Analyzer. Western Blot results are shown in Figure 12.



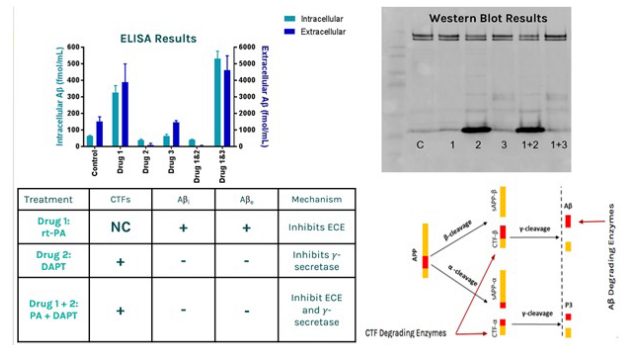
**Figure 12:** Western Blot results.

The Western Blot result in Figure 12 corresponds to Control, Drug 1, Drug 2, Drug 3, Drugs 1 & 2, and Drugs 1 & 3 (left to right). Ladder is visible on the left. Western Blots detects the concentration of C-terminal fragments (CTFs). On an APP protein, the top amino acid on the amyloid-beta sequence is known as the N-terminal whereas the bottom amino acid on the sequence is known as the C-terminal. The C-terminal fragments are the chains of amino acids in between the C-terminal and the end of the APP protein. As shown in the Western blot result in Figure 12, Drug 1 appears to have no impact at all on the production of CTFs as the column for the control and Drug 1 are identical. On the other hand, Drug 2 and Drugs 1 & 2 in conjunction evidently show an increased number of CTFs. Drug 3 seems to have increased the number of CTFs as its column is not completely identical with the control. The same can be said for the combination of Drugs 1 & 3.

### Conclusions and Discussion

From the Western Blot results, it was observed that the Activase® rt-PA (Drug 1) did not affect the CTF concentration, however, the sample with this drug exhibited increased A $\beta$  concentration as portrayed by the ELISA test. Because of this, it is concluded that alteplase has no effect on CTF degrading enzymes. Rather, the alteplase inhibited the A $\beta$  degrading enzyme called the endothelin-converting enzyme (ECE). By inhibiting the ECE, the amyloid- $\beta$

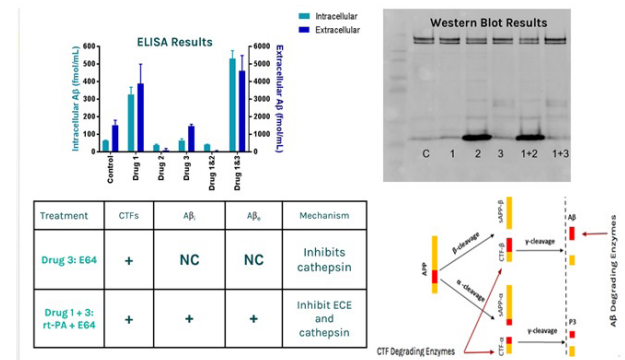
peptides were not being efficiently degraded thus causing an increase in A $\beta$  concentration. Hence, this increased concentration would have caused an accumulation of amyloid- $\beta$  peptides and may form neurotoxic plaques which are known to trigger neuronal loss.



**Figure 13:** Combined conclusion from ELISA and Western Blot test on efficacy of Activase® rt-PA (Drug 1) and DAPT (Drug 2).

Unlike the alteplase, DAPT (Drug 2) increased the concentration of CTFs, but decreased the concentration of amyloid- $\beta$  peptides. Therefore, it can be concluded that DAPT inhibits  $\gamma$ -secretase. This is the enzyme which cleaves the CTFs into the amyloid- $\beta$  peptides, however, if this enzyme is inhibited, the amyloid- $\beta$  peptides cannot be formed. This explains why there was an accumulation of CTFs and a decrease in amyloid- $\beta$  peptides. This results in a desirable condition as this would prevent the accumulation and formation of amyloid plaques, and extreme neuronal loss can be prevented.

When combining these two drugs, both the ECE and the  $\gamma$ -secretase would be inhibited, however, the data for the combination of these drugs does not show the inhibition of ECE since the amyloid- $\beta$  peptides were not being produced in the first place due to the inhibition of  $\gamma$ -secretase.



**Figure 14:** Combined conclusion from ELISA and Western Blot test on efficacy of clonazepam (Drug 3).

For the clonazepam E64 (Drug 3), the Western blot test showed a slight increase in CTFs, however the A $\beta$  concentration was unaffected. Since A $\beta$  production continued as normal, it means that the  $\gamma$ -secretase was not affected and continued to cleave the amyloid- $\beta$  peptides. Therefore, the conclusion is drawn that the clonazepam inhibits the CTF-degrading enzyme called cathepsin. Because of this, the CTF concentration was slightly greater than

normal.

When clonazepam E64 was mixed with Activase® rt-PA, both the ECE and cathepsin were inhibited. Because of this, CTF levels increased slightly, and due to the inhibition of ECE, the concentration of amyloid- $\beta$  also increased. These conditions would lead to the accumulation of neurotoxic plaques which is detrimental to the patient with Alzheimer symptoms.

In conclusion, from this research investigation, it is determined that DAPT (unaccompanied with another drug) is effective in preventing the accumulation of amyloid- $\beta$  polypeptides because of its inhibition of  $\gamma$ -secretase. This confirms the efficacy of DAPT. Although the inhibition of  $\gamma$ -secretase prevents the formation of amyloid plaques, the complete inhibition of  $\gamma$ -secretase can be harmful to the human brain in other ways. The  $\gamma$ -secretase is not an enzyme specific to APP and thus has other beneficial uses in the brain [6]. This study does not investigate the side effects of the inhibition of  $\gamma$ -secretase, but the results of this study can be further used to examine alternative results of the inhibition of  $\gamma$ -secretase.

### Acknowledgments

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