TARGETED CANCER THERAPIES; CREATING AND PURIFYING A MUTATED VERSION OF CARBOXYPEPTIDASE-A TO ACTIVATE CAPPED METHOTREXATE FOR USE IN ANTIBODY-DERIVED ENZYME PRO-DRUG THERAPY (ADEPT)

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ABSTRACT: Glioblastomas currently make up 45.2% of all malignant brain tumors and have an extremely poor survival rate due to lack of effective treatment options. The following study examines the possible application of ADEPT (Antibody Directed Enzyme Prodrug Therapy) as a treatment for glioblastomas. This method would allow for on-site activation of an inactive, masked pro-methotrexate molecule (FDA approved) by a mutated Carboxypeptidase A (mCPA) enzyme. Designing for selective activation of the methotrexate at the source of the tumor allows for directed cancer treatment while substantially reducing side effects. Under the supervision of Mike Dorrell, PhD and Heidi Woelbern, PhD, a point mutation for the active site of CPA was designed to generate an expression construct with the mutated version of CPA. This point mutation expands the active site allowing for the extra large amino acid-like 'mask' of pro-methotrexate to be cleaved, and the methotrexate drug thus activated. This point mutation of a natural human enzyme prevents rejection and is specific enough that endogenous wild type enzymes will not activate the drug. HEK 293 cells were transfected with the construct to produce the enzyme. In this study, transfection protocols were optimized and purification processes were tested and optimized for protein production and identification.

Glioblastomas are an extremely devastating form of cancer that do not currently have viable treatment options available. Most primary malignant brain tumors (45.2%) each year and 54.4% of all gliomas are found to be glioblastomas. These tumors are present in nearly 15,000 cases in the U.S. a year and have an incidence rate of 3.19 in 100,000 people [1,2].

While there are many types of glioblastoma histologies, the most aggressive and deadly form is the glioblastoma multiforme (GBM). Once the patient is diagnosed, the average survival time is between 10-12 months, with less than 5% surviving beyond 5 years[3,4]. Since the blood-brain barrier prevents toxins from entering the brain, chemotherapy has proven nearly ineffective as the dose required to kill the tumor becomes lethal to the body long before the tumor is impacted. Brain surgery has also been examined to remove the tumor. However, brain damage becomes extremely common when this procedure is carried out and it is extremely difficult to remove the entire tumor due to the non-uniform tumor shape that includes multiple finger-like extensions and small, individual tumor sites that have broken away from the main tumor. Despite over a century of research and progress being accomplished in many fields, including neuro-oncology

and diagnostics, the mean survival rate of GBM patients has increased by less than one year. Since a need still exists, novel forms of treatment must be explored[5,6].

ADEPT: Antibody directed enzyme prodrug therapy (ADEPT) is a treatment strategy of interest especially for glioblastomas. This therapy would allow for chemotherapy to be targeted specifically to the tumor site in the brain. The treatment strategy would allow for a masked and non-toxic form of the chemotherapy drug to be taken into the body and systematically activated only at the site of interest by an enzyme (*Figure 1*). A site of interest, such as a glioblastoma, can be targeted by identifying tumor specific markers, which historically has been an issue for cancer applications. Once a marker has been identified, an antibody specific to the target is chosen. This antibody will be the compliment to the target and will not bind at non specific locations throughout the body. This antibody will be bound to an enzyme which will activate the prodrug used for chemotherapy into the active form of the drug at the target site (*Figure 1*). By utilizing this strategy for cancer treatment, a high degree of specificity could be achieved while limiting the unwanted side effects commonly encountered during chemotherapy[7] (unpublished results in our lab).

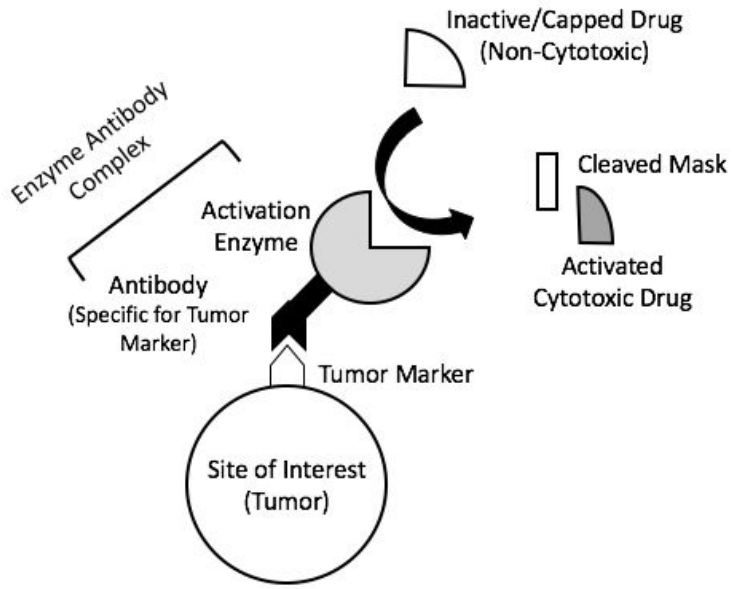

Figure 1. Antibody Directed Enzyme Pro-Drug Therapy (ADEPT) Model. A marker specific to the site of interest (Tumor) becomes the main target for an enzyme-antibody complex that has been specifically engineered to mimic endogenous enzymes and activate a masked drug into its active cytotoxic state

Historically, ADEPT has had two major hurdles that have prevented its success as a viable treatment. 1) Identification of a tumor-specific target for which the antibody can bind and thus localize specifically to the tumor has proven very difficult. This is largely because tumor cells are abnormal versions of normal cells within the body. Thus, while levels of markers expressed on the tumor might be slightly different, or some markers may have small, but important mutations, the markers are not distinct from the normal tissue cells and thus targeting them is difficult. 2) Second, identification of an enzyme and pro-drug combination that only causes the drug to become activated at the site of the tumor is difficult due to the tendency of the body to reject foreign enzymes, while normal enzymes are naturally expressed at varying places in the normal, healthy body. Our strategies to overcome these two obstacles are discussed below.

TAM's: Tumor specific markers have commonly been a problem for ADEPT since cancer cells express many of the same markers as healthy cells. However, the unique micro-environments within cancers provide the answer to the problem. The strategy in our lab is to target the tumor helper cells rather than the tumor cells themselves. Glioblastomas secrete chemicals which allow them to take over immune macrophages (the body's main source of defense against cancer) in order to convert the macrophages from killer macrophages that eliminate foreign or unhealthy cells (M1s) to macrophages that promote cell growth (M2s). Once these macrophages are turned into tumor associated macrophages (TAM's), an extreme version of the body's normal M2 macrophages, they help provide an environment conducive to tumor growth while simultaneously providing protection from the body's immune system. When the cells are altered by the tumor to become TAMs, they begin to express mannose macrophage receptors (MMR) on their surface. Within the brain, MMR is only expressed on TAMs within the tumor microenvironment and not on the brain's normal macrophages or microglia. Thus it provides a specific target that can be used for ADEPT (*Figure 2*)[8].

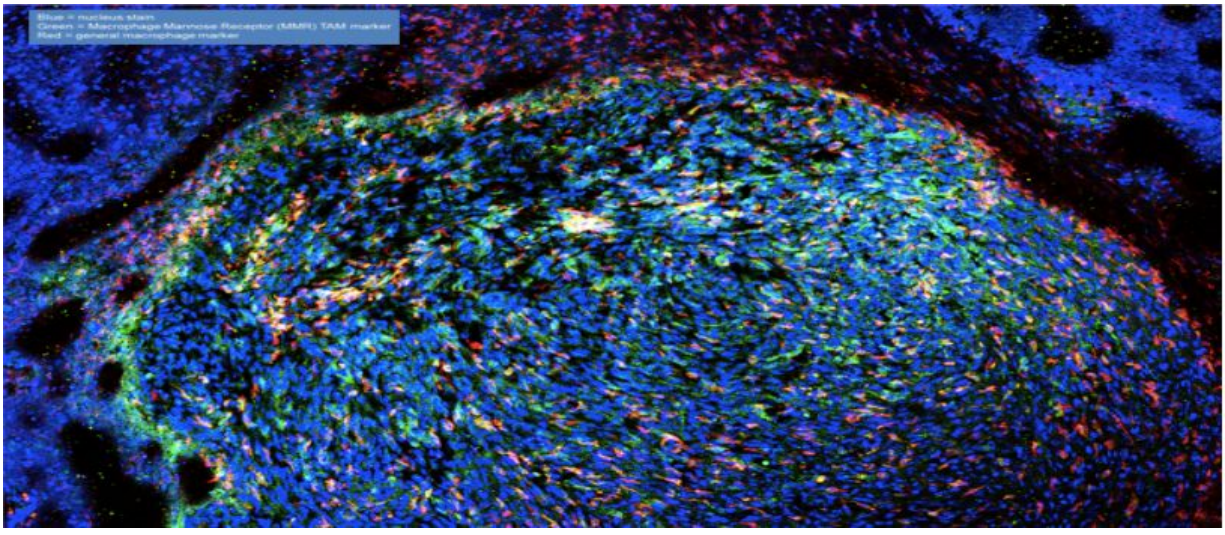

Figure 2. The presence of macrophage mannose receptors (MMR) around tumor cells seen under

fluorescent microscopy. Normal macrophages are seen in red, cell nuclei are seen in blue, and MMR appear green in the image. Dense clusters of MMR are observed surrounding the exterior of the tumor. MMR are not seen in other locations in the body, thus they appear to be a good target/marker for ADEPT therapy.

Enzyme-Prodrug Combination: Activation of the prodrug must occur only at the site of interest, marked by MMRs within the tumor microenvironment. Thus, activation must occur solely by the enzyme linked to the MMR-targeting antibodies and not by various naturally occurring enzymes scattered through the body. Because of this, using an enzyme that is not endogenous to the human body would make sense for this targeted therapy. However, the body's immune system attacks foreign enzymes and a non-endogenous enzyme would be immediately rejected. On the other hand, using naturally occurring human enzymes, while eliminating the problem of immune rejection, will likely cause activation of the prodrug at other, non-tumor sites. One main approach is to alter the active site of a naturally occurring enzyme to solely activate the prodrug (*Figure 3*). Small mutations to naturally occurring enzymes are generally not sufficient to elicit an immune response since these mutations are not recognized as foreign. However, if the mutation sufficiently alters the active site such that the mutated enzyme is now the only version that can cleave, and therefore activate, the pro-drug, then specificity can be achieved. Carboxypeptidase A is a naturally occurring pancreatic enzyme which cleaves bonds near the c-terminus of amino acids with aromatic (or aliphatic) side chains. As such, masking a pre-existing drug to be activated by this mechanism holds a large amount of potential. As long as the the mask resembles the side chain that the molecule typically cleaves, then it can be activated by the enzyme at the desired location[9]. Our strategy is to coordinate a point mutation in the carboxypeptidase A enzyme that permits selective activation of an amino-acid like mask added to methotrexate.

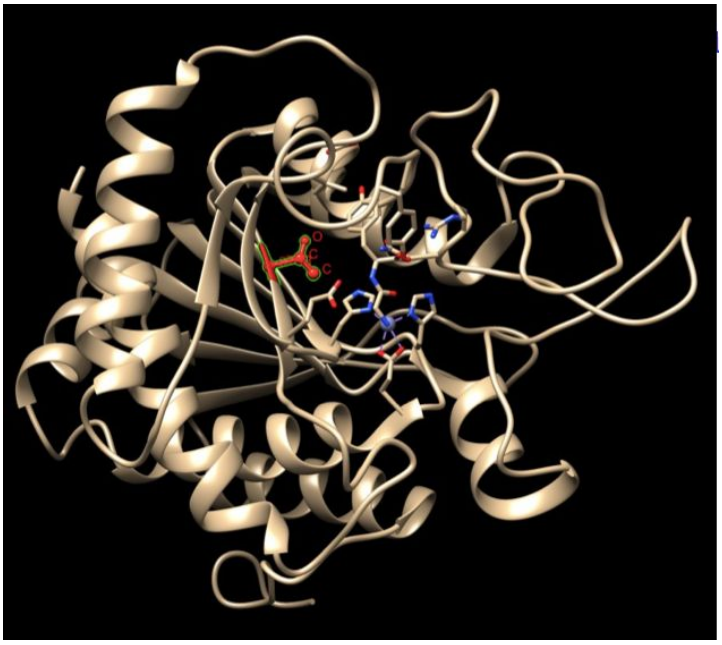

Figure 3. Active site of Carboxypeptidase A (CPA) showing the amino acid (Threonine) which has been selected to undergo a point mutation to Glycine to create a larger active site. The prodrug is designed to be selectively activated by the mutated CPA. This version of CPA will not activate molecules in the body.

Methotrexate is a FDA approved chemotherapy drug designed to target rapidly dividing cells in the body. Although very effective at killing rapidly dividing tumor cells, it also attacks other cells in the body which are rapidly dividing, causing a number of side effects. Activating this drug only at the desired site would allow for a larger amount of Methotrexate to attack the tumor cells without the unwanted side effects on healthy cells throughout the rest of the body. In order for CPA to activate a prodrug version of methotrexate, the cap must resemble the amino acid structure on c-terminus which CPA typically cleaves. However, it is also essential that that the prodrug is not activated by endogenous CPA (wtCPA). Thus the mask must be slightly larger than the average side chain so it will not fit into the active site of wtCPA (*Figure 4*).

Coordinating mCPA with pro-methotrexate: The pro-drug moiety of pro-methotrexate is an amino acid (observed at the left of the blue-bracketed pro-drug moiety) with an extra-large side chain (at the right of the chemical pro-drug moiety) (Figure 4). This amino acid is larger than any of the twenty naturally occurring amino acids. Thus, it will not fit in the active site of normal, endogenous CPA meaning that naturally occurring CPA within the body will not activate the pro-drug. However, the point mutation introduced in mCPA (figure 3), opens the active site by eliminating the red side chain that normally resides within the active site. This mutated version is now specifically able to cleave the larger amino acid that constitutes our pro-drug moiety on methotrexate.

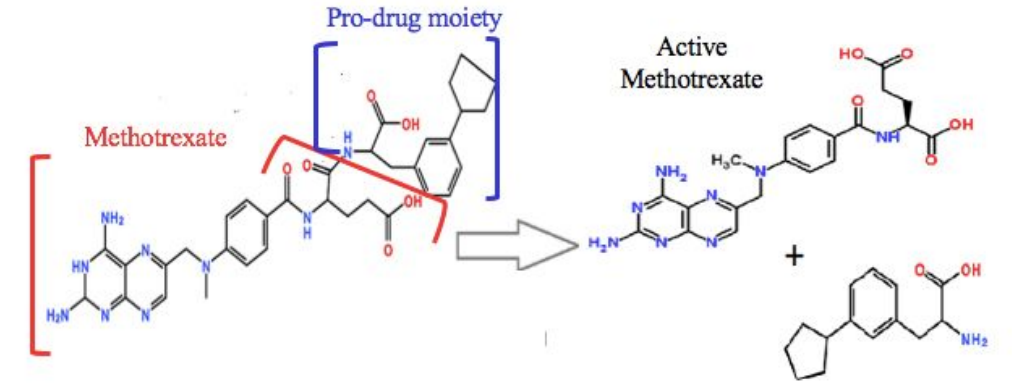

Figure 4. Methotrexate is a chemotherapy drug designed to target rapidly dividing cells. Adding a cap to methotrexate which resembles tryptophan (an aromatic amino acid) will allow for cleavage and activation by a mutated form of CPA.

ADEPT includes three major steps before it can be approved for clinical studies. 1. A mutant form of CPA must be created and shown *in vivo* to cleave the pro drug without cleaving other essential elements. 2. The antibody-enzyme complex must show *in vivo* to attach only to tumor sites via MMR targets on glioblastoma cells. 3. Lastly this therapy must be inserted directly into the brain to avoid blood-brain barrier dissociation and must be tested in *in vitro* studies. This study focuses on the first step of ADEPT by creating and isolating the mutant form of CPA.

MATERIALS AND METHODS:

Site Mutagenesis. Human Carboxypeptidase A1 (CPA) DNA was acquired from Sino Biologicals (Beijing, China) Two point mutations were induced in the active site of CPA to change threonine 268 to glycine (T268G) using a QuikChange II XL Site-Directed Mutagenesis Kit from Agilent Technologies (La Jolla, CA). This point mutation was made on forward and reverse primers by changing ACC to GGC (a1132g, c1133g). The primers were ordered from Agilent Technologies (La Jolla, CA). An 18 cycle PCR reaction occurred to acquire the forward and reverse primers to cause the point mutations. Using a DPN I digest, all DNA that had undergone the PCR reaction was preserved. This DNA was next transformed into XL10-Gold Ultracompetent Cells from Agilent Technologies (La Jolla, CA). These cells were incubated for 16 hours at 37 C. After the 16 hour incubation period, five colonies were chosen to grow up in LB broth. A mini prep of the DNA from each of the colonies was done using a QIAprep Spin Miniprep Kit (250) by Qiagen (Hilden, Germany).To determine DNA concentration in each of the samples, a NanoDrop 2000c by Thermo Scientific (Waltham, MA) was used. Additionally, each of the samples were sequenced to determine if the point mutations occurred. After the sequencing, all but 1 colony incorporated the point mutations. The mutated DNA from colony 5 was used to make a maxiprep from.

Transfection. In order to produce large quantities of the CPA protein, T-75 polylysated flasks of Human Embryonic Kidney cells (HEK293) were transfected with mutant CPA (mCPA) DNA. An additional flask was transfected with wild type CPA (wtCPA) as a control. Before transfection, the confluency of the HEK cells was determined to be between 40-50%, which was required for proper transfection. A transfection kit was obtained from Invitrogen (Carlsbad, CA) which utilized a lipofectamine reagent and the standard Invitrogen kit protocol was performed on the HEK cells. After 24 hours of incubation, Halt Protease Inhibitor Single Use Cocktail, EDTA-free (100x) by Thermo Scientific (Rockford, IL) were used to prevent proteolytic degradation during cell lysis and purification. The cells were then left to incubate for an additional 24 hours. After the total incubation time of 48 hours had passed, the cells were harvested for purification and analysis. To harvest, the supernatant was first collected and stored. Then, the cells adhered to the plate were lysed using M-PER reagent to release any protein

within the cells and the lysate was collected. Both the lysate and the supernatant were used for purification.

Purification. The first design for purification was using the Pierce c-Myc Tag Magnetic IP/Co-IP Kit by Thermo Scientific (Rockford, IL).In this protocol there were anti-c-myc antibodies attached to magnetic beads. The magnetic beads were prepared by doing washes with TBST. Next, the lysate and supernatant from the different conditions were placed in eppendorf tubes with the anti c myc magnetic beads and 400 µl of TBST. When the lysate and supernatant were incubated with the beads over a period of 30 minutes, the CPA proteins (that are tagged with c-myc) theoretically should have bound to the beads. After the incubation period concluded, the beads were gathered using a magnetic stand and were washed three times with TBST and one time with ultrapure water. The proteins attached to the beads were eluted using a gentle elution protocol. This elution process used c-myc peptide at 0.5 mg/ml in TBS. 100 µl of 0.5 mg/ml c-myc peptide in TBS was applied to each of the magnetic bead purification. This was allowed to incubate at 37 C while rotating for 10 minutes. The magnetic beads were then collected using a magnetic stand and the supernatant was collected for analysis. Analysis of the purified CPA proteins was done using a Bradford Assay and Western Blot.

Assays. A Bradford Assay was done on the bound to bead and unbound to bead supernatants and lysates of both the mCPA and wild type CPA. This Bradford Assay ran at a wavelength of 595 nm and determined that there were proteins in each of the samples. Because there were proteins present, a Western Blot was done to determine if the CPA was among those proteins.

Two Western Blots were done on the bound to bead and unbound to bead supernatants and lysates. The Western Blot was prepared using 30.5 µl samples using 20 µl samples from transfection, 7.5 µl of LDS and 3 µl of reducing agent. The samples were put in a 90 C bath for 10 minutes. Samples were loaded in a 12% gel. After loading the sample 1 ml of antioxidant was applied on top of the loading wells. The gels were set up right in the apparatus. The gels were ran at 200 Volts for 2 hours at 100-150 mAmps. Transfer buffer was made consisting of 50 ml Bolt Transfer Buffer (20X), 1 ml of nupage Bolt antioxidant, 100 ml methanol, and 849 ml milliQ water. Enough transfer buffer was placed in small plastic containers to cover the gels when released from their compartment. Sponges for the blot were soaked in the transfer buffer. The gels were released from the compartments and placed on the sponge stack. PVDF membrane paper was soaked in methanol for 30 seconds then placed on top of the gel accompanied by another sponge. All bubbles were rolled out prior to running the blot. The blot was set in the apparatus and ran at 20 V for 1 hour and 260 mAmps. After the blot was complete, the membrane paper was removed and washed twice with ultrapure water. The membrane paper was left overnight in 5% milk buffer + TBST at 4 C. The next morning, primary antibody in 5% milk buffer + TBST was added to the membrane. This was allowed to sit for 2 hours. The membrane was then washed with TBST 6 times. The secondary antibody in 5% milk buffer + TBST was next added to the membrane and allowed to sit for 1 hour. For each antibody addition, the membrane was kept in a shaking apparatus at 4 C. The secondary antibody was then washed with TBST 6 times. The Blot was analyzed under a UV spectrometer. Both Western Blots resulted in inconclusive results. Because of this, steps were taken to optimize protein formation and purification.

Optimization. In order to test transfection efficiency with the HEK cells, a set of different transfection conditions were created to optimize the most protein synthesis. For this optimization a Mammalian Beta-gal Assay Kit by Thermo Scientific (Rockford, IL) was used. Beta Galactosidase DNA was transfected into HEKs in a polylysated 12 well plate and different amounts of DNA relative to the lipofectamine reagent. The amount of Beta Galactosidase protein that was synthesized was read by harvesting the cells using Beta-Galactosidase Assay Reagent, letting the reagent and cells incubate for 30 minutes, and then measuring the absorbance of protein at a wavelength of 405 nm on a plate reader. Results showed that using 2.4 µl lipofectamine reagent for every 2000 ng of DNA resulted in the largest amount of protein synthesis.

Transfection Part II. After the optimization, the same conditions of 2.4 µl of lipofectamine reagent for 2000 ng of CPA (wild type and mutant) DNA was used to transfect into HEK cells. HEK cells were once again added to polylysated T-75 flask and were transfected using the optimized conditions. At the 24 hour mark, 100x Halt Protease was added to each flask. At the 48 hour mark, the supernatant of both the wild type and mutant CPA was collected and saved for purification. The remaining adhering cells were then lysed adding 1 ml of M-PER Mammalian Protein Extraction Reagent from the Mammalian Beta-Galactosidase Assay kit. The cells were shaken with the reagent for 5 minutes and then collected and saved for purification.

Purification Part II. In this purification, new measures were taken to better purify the CPA proteins. Affinity Column Chromatography was used. Columns were packed in disposable 5 ml Polypropylene Columns by Thermo Scientific (Rockford, IL) using Pierce Control Agarose Resin for the control column and Pierce Anti-C-myc Agarose Resin by Thermo Scientific (Rockford, IL) for purification of the wild type and mutant CPA. The columns were packed by using degassed PBS buffer for washing. A bottom porous filter was placed inside the disposable column. Next 4 ml of resin was applied to each designated column. Two anti-c myc columns and one control column were made. The resin was allowed to sit and equilibrate in the column for 30 minutes. After 30 minutes, the column was unplugged and allowed to drain until liquid barely covered the top of the resin bed. A second porous filter was added on top of the column bed. The columns were stored with 0.12% sodium azide in PBS at 4 C. In order to purify using the columns, the columns were first equilibrated to room temperature and washed with 2 5ml volumes of TBS. Next the 1 ml lysate samples of both the mutant and wild type CPA were placed on separate anti-cmyc columns and the flow throughs were collected. The control resin column used lysate from the wild type CPA. After the lysate was ran through on the column, 10 2 ml washes of TBS-T occured. To elute the proteins off of the column, a chemical elution protocol using 3M NaSCN was used. 1 ml increments of 3M NaSCN were added to the column for a total of 3 elution fractions. (3 ml of 3M NaSCN per column was used) Each fraction was collected in a separate tube. After the elution of the lysate proteins, the columns were washed with 10, 2 ml increments of TBS-T. The procedure was followed by using the supernatant. (10 ml supernatant of mutant CPA on anti c-myc column, 5 ml supernatant of wild type CPA on anti c-myc column, and 5 ml supernatant of wild type on control column) After the elution of the supernatant the columns were washed again with 10, 2 ml increments of TBS-T. The columns were then stored in 4 ml of TBS-T + 0.05% sodium azide at 4 C for repeated purifications.

RESULTS AND DISCUSSION:

Miniprep. Carboxypeptidase A DNA concentrations from 5 colonies that underwent point mutations from ACC TO GGC (threonine to glycine). The highest DNA concentration was observed in the control and colony 3 (*Figure 7*). This suggests that all colonies had successful incorporation of the CPA and mCPA plasmids. These colonies were all used to make the proteins for further testing and analysis.

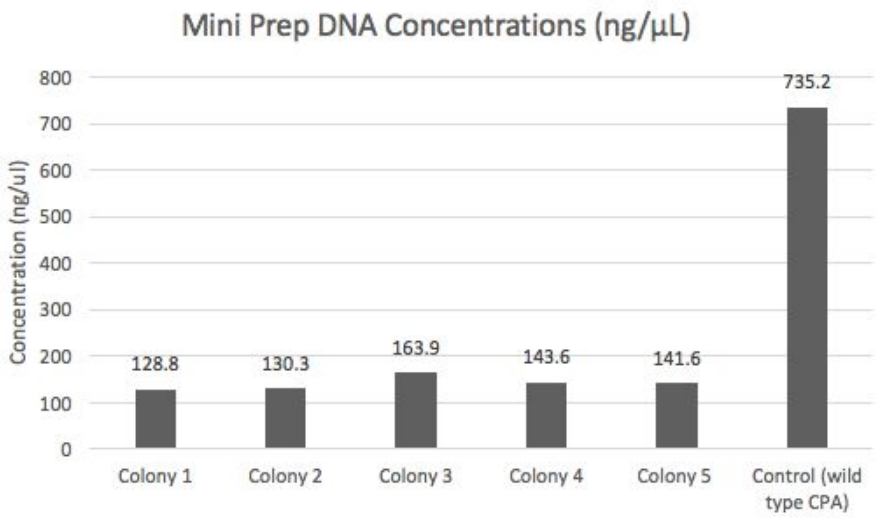

Figure 5. DNA Concentration Determination from Mini-Prep. Carboxypeptidase A DNA concentrations(ng/ul) from 5 colonies which potentially underwent point mutations from ACC TO GGC (threonine to glycine), the colonies were purified via mini prep in order to be prepped for sequence analysis.

Sequencing. After mini preps of colonies 1-5 were done, all 5 colonies were sequenced to check for incorporation of two point mutations (a1132g, c1133g). Colony 5 resulted in the incorporation of both point mutations and the resulting DNA sequence presented with the highest purity. Further maxiprep and experiments on mutant CPA DNA was done using the DNA from Colony 5 (Figure 8). Thus, cells making the mutant CPA had been successfully generated (colony 5) along with cells producing the wild-type version of human CPA (control) for use in generating and purifying the desired enzymes.

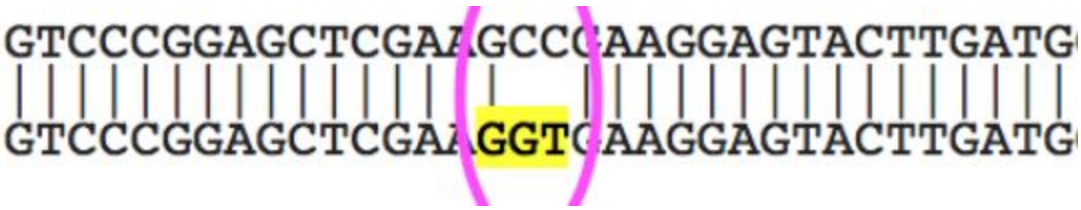

Figure 6: Sequencing Results of Colony 5. Sequencing results of Maxiprep colony five demonstrating the incorporation of 2 necessary/desired point mutations (a1132g, c1133g). The highlighted sequence shows the wild type sequence of CPA, two point mutations occurred changing an adenosine and cytosine both to guanines (ACC to GGC) **Note this is the Reverse Strand Sequence therefore for the reverse strand the double point mutations changed a guanine and threonine to two cysteines (GGT to GCC).

Bradford Assay. A Bradford analysis measured the absorbance at 595 nm using a Synergy Multi Mode Plate Reader to detect protein presence of samples taken after purification using the magnetic bead protocol. The standards were used to generate a best fit line to relate absorbance at 595 nm to protein concentration. Once the best fit line was obtained: y=0.0004x+0.3785, the absorbances of each sample were used to determine the concentration of protein in µg/ml. Absorbance of proteins were observed in all mCPA and wtCPA wells. The concentrations of proteins for each sample were: Wild Type CPA Lysate (WT-L)-2193.125 µg/ml, Mutant CPA Lysate (MUT-L)- 1875.625 µg/ml, No Plasmid Lysate (NP-L)- 2735.625 µg/ml, Wild Type CPA Supernatant (WT-S)- 3698.125 µg/ml, Mutant CPA Supernatant (M-S)- 3105.625 µg/ml, and No Plasmid Supernatant (NP-S)-2443.125 µg/ml. (*Figure 7*). Further testing is required to confirm the identity of the protein.

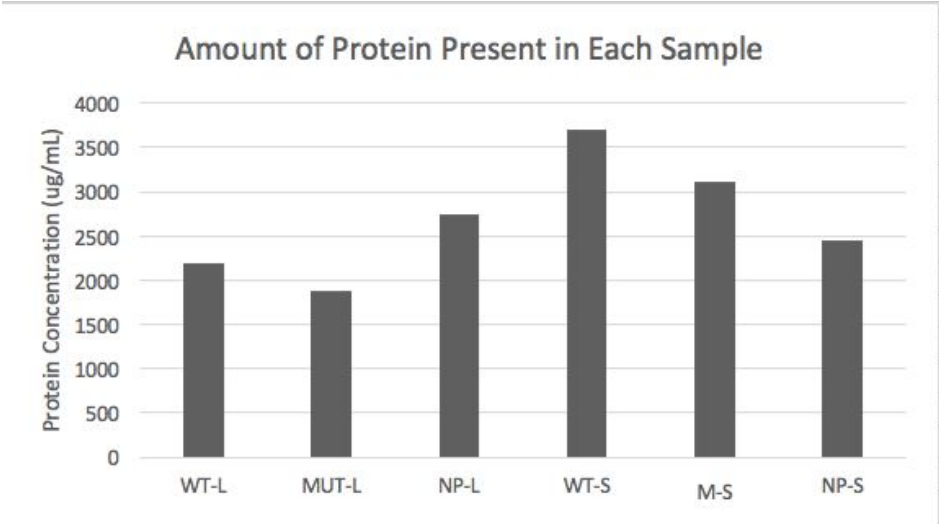

Figure 7. Bradford Analysis to Determine Protein Concentration. Bradford Assay Generation of protein concentration standard curve and analysis of WT, Mutant, and no plasmid samples. A bradford assay was run at 595 nm using a NanoDrop. Protein concentration of the samples was determined from

the standard curve and the best fit line was determined to be y=0.0004x+0.3785. The absorptions obtained from each sample were imputed and the concentration was calculated for each sample.

Western Blot. The protein of interest has a molecular weight of 37.280 kDa and possesses a C-Myc tag, making anti C-Myc antibodies the ideal antibodies for viewing the protein on a western blot. The C-Myc tag is added to the CPA (wt and mutant)genes as part of the protein sequence, so the bulk of the C-Myc tagged protein, so there should be no signals observed in the no plasmid samples. Since CPA expresses a secretion signal sequence as part of the N-terminal sequence, it is expected that the protein will be found in the secreted/supernatant media, However, with high degrees of transfection, the protein would also show up in the lysate samples in higher concentrations, thus the lysate samples are run on the western blot.

The major bands at 26 and 43 kDa can be eliminated since appear in the no plasmid sample, which should not possess the protein. Additionally, the protein of interest is known to occur possess a molecular weight around 37 kDa, which is promising since unique bands appear around that molecular weight. However, the uncertainty appears when the bands around that region are examined. If purification had occured properly, the bound samples should show primary expression of the protein, with the unbound showing only trace amounts (*Figure 8*). Since this is not the case, the western blots are inconclusive and suggest that the purification method used was not ideal or the transfection did not occur properly.

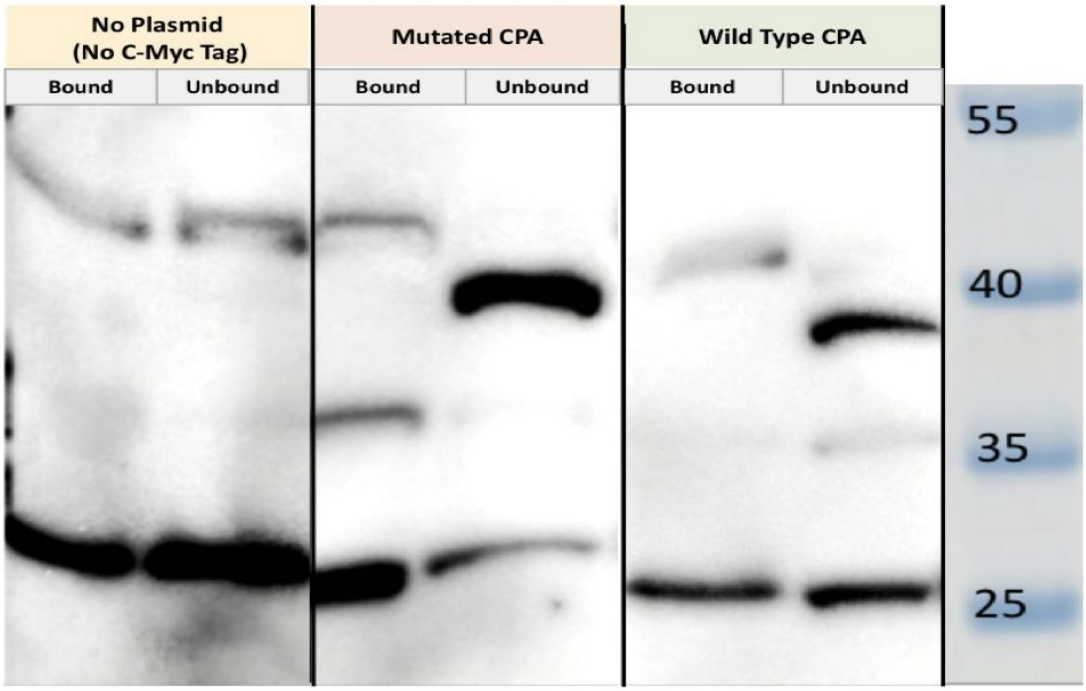

Figure 8. Inconclusive Western blot stained with Anti-C Myc antibodies. Samples of purified

proteins obtained from the lysate of the wild type, mutant CPA and no plasmid (No C-Myc Tag) transfected cells were taken and used to compare the efficacy of the purification technique (Magnetic beads) by comparing the protein found within the unbound medium and bound medium. If purification occurred properly, the most definitive band would be expected to occur around 35 for the bound samples of Mutated CPA and Wild Type CPA. Inconclusive results were obtained due to lack of definition in the band appearance in the bound categories suggesting purification inadequacy.

Transfection Optimization Assay. The identity of the proteins could not be inferred from the western blot. This lead to optimization of the transfection protocol through a Beta Galactosidase assay as well as alteration of the purification protocol through a Beta Galactosidase Assay.

Beta Galactosidase was transfected into HEK293 cells under various concentrations of Lipofectamine (Lipo) reagent and Beta Galactoside DNA were tested to find the optimal transfection conditions. Absorbance at 405 nm is proportional to the amount of protein produced by the cells. The highest absorbance readings were absorbed in the 200 ng of DNA condition with 2.4 ul of Lipofectamine (*Figure 9)*. These conditions were chosen for all future transfections performed. It should be noted that the conditions we had previously been using for the transfections did not show significant levels of beta galactosidase expression suggesting that we were not operating at the optimized conditions and may not have been effectively transfecting the cells with our CPA plasmids.

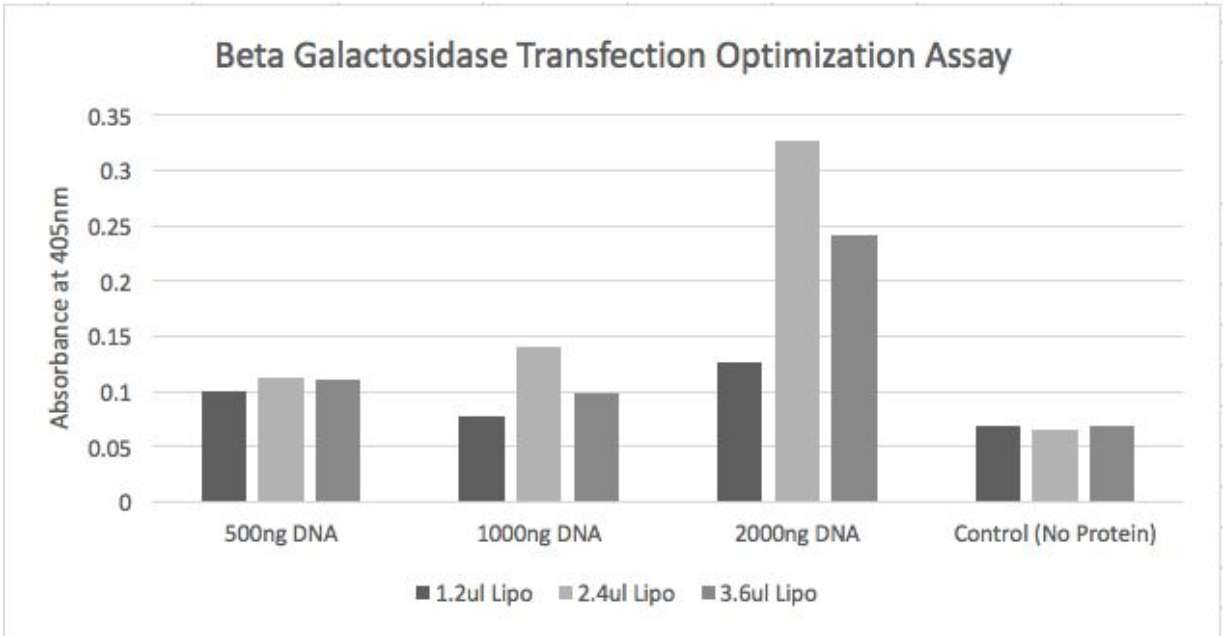

Figure 9. Beta Galactosidase Transfection Optimization Assay Transfection conditions with various levels of Lipofectamine (Lipo) reagent and Beta Galactoside DNA were tested for find the optimal conditions for HEK293 cells. The highest absorbance readings were absorbed in the 200 ng of DNA condition with 2.4 ul of Lipofectamine.

Western Blot with New Protocol. Using optimized transfection conditions, purification was attempted using columns. The columns were packed with control beads or anti-cmyc resin and a slow flow was achieved using gravity to pull the samples over the beads. It is anticipated that this method will allow for better purification of the supernatant, which typically contains 10-15 mls of liquid. The larger surface area of the columns allows for more of the supernatant to come into contact with the anti-cmyc tags than the beads allowed for.

To check the efficacy of the new transfection and purification protocol, an anti C-Myc western blot was run. Only the wild type transfection lysate was run to provide a baseline for further western blot analysis. Agarose beads served as a control and Anti-C-Myc beads served as the method of intended purification. Samples were taken before any purification occured (Pre Run), and once on the columns: the flow through and the elution portions of the purification for each column.

Pre-Run, as expected, showed a large mass of proteins around the region of interest. When examining the control beads, the band around 40 appears only in the flow through and not the elution, this is also expected considering binding of the protein of interest to the beads should not occur. The Anti-cmyc column results were also promising as a distinct band was observed in the elution fraction, suggesting the protein of interest is not only present, but properly binding to the column beads. The presence of a band around the same mw suggests the need for further optimization of the purification protocols (*Figure 10*).

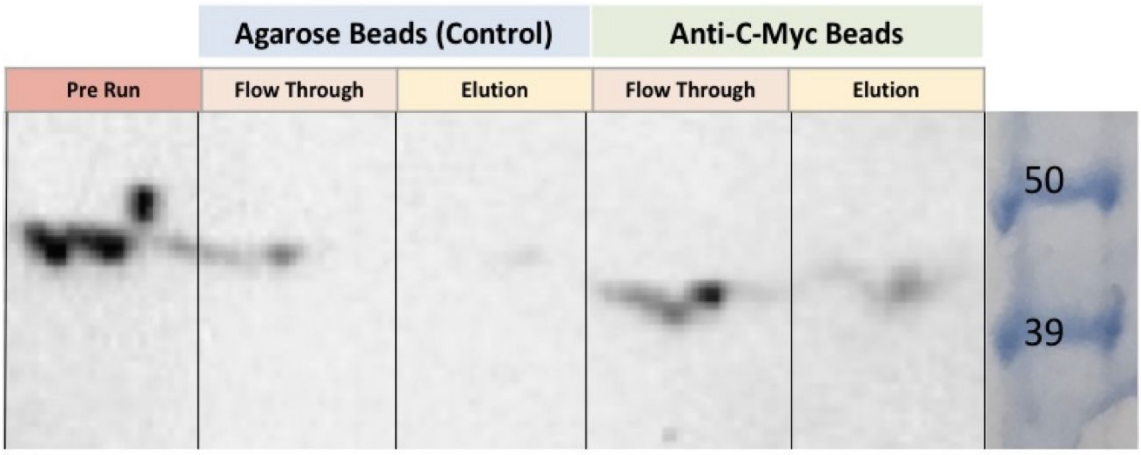

Figure 10. Conclusive Anti C-Myc Western Blot from Column Chromatography. To check the efficacy of the new transfection and purification protocol, an anti C-Myc western blot was run. Agarose beads served as the control and did not show binding in the elution fraction, which was expected. Anti-cmyc beads did show binding in the elution fraction around the correct molecular weight, suggesting that the protein of interest has been obtained. Further purification optimization is necessary to lessen the band seen in the Anti-cmyc flow through.

Optimization of transfection protocols alongside changing the purification method provided fruitful results. The latest western blot suggests that the protein of interest is being purified by

the anti-cmyc resin in the columns. Further plans include running the mutated transfection on a western blot as well as optimization of the purification. Ideally, no band would be observed in the flow through, and all the protein would be found in the elution fraction for the anti c-myc beads.

CONCLUSION:

To accommodate for the large prodrug molecule needing activation for ADEPT, Point mutations to increase the active site of Carboxypeptidase A were designed and successfully incorporated into the genetic code of a plasmid. Transfection and purification protocols were carried out to generate and isolate protein. A Bradford assay was used to assess the amount of protein produced by HEK 293 cells transfected with WTCPA, MutCPA, and no plasmid suggesting that protein was being properly produced. However, the identity of the protein was not confirmed due to inconclusive western blot results. Transfection protocol for HEK293 cells was then optimized using a Beta-Galactosidase assay and new conditions of Lipofectamine and DNA were chosen. After a large amount of troubleshooting and analysis, it was determined that magnetic beads were not the most effective method to purify the protein of interest due to such large volumes of samples that needed purification. It is anticipated that column purification will greatly optimize the purification process as it allows for the anti-c-myc tag to interact with a larger volume of the sample requiring purification.

Further project plans include identification of the protein produced by using a western blot as well as externally running mass spectrometry analysis, and other analytical analyses, of the isolated protein to confirm the identity. Once the identity is confirmed the purification process will undergo optimization and the process will be scaled up.

Furthermore, viability assays on tumor cells in culture will be used to test the enzyme's ability to activate pro-methotrexate. Methotrexate (positive control) should successfully kill the cells whereas treating the cells with the pro-methotrexate (negative control) will leave the cells unharmed. Pro-methotrexate and control CPA (wtCPA) should also leave the cells unharmed, since the wtCPA should not activate the pro-methotrexate. However, the combination of pro-methotrexate and mutant CPA (mCPA) should prove lethal to the cells if our enzyme and pro-drug combination is working as designed; the mutant CPA will cleave the “mask” from the pro-methotrexate, thus rendering it the same as active methotrexate, but only in the presense of mCPA. Once the interaction and activation is confirmed, the mCPA would be linked to MMR antibodies and the ADEPT system would be tested within cultured cells. These studies should prove to be a vital step on the way to testing the entire system in vitro, and then in vivo. If all works as hypothesized, this may be a promising method for treating glioblastomas without the standard side effects of traditional chemotherapy.

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