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Matthew Karpen March 25th, 2025

Humanization of lead ancestral L-asparaginase candidates identified through ancestral sequence reconstruction

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Abstract

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Acute lymphoblastic leukemia (ALL) is the most prevalent form of pediatric cancer. Current chemotherapy treatments utilize a bacterial L-Asparaginase (L-ASNase) derived from either *Escherichia coli* or *Erwinia chrysanthemi*. Without the L-ASNase treatment, the patient's chances of remission decline. ~30% of patients have an immunological response to the bacterial L-ASNases, so research for another less immunogenic asparaginase is warranted. Previous research established the effectiveness of the *Cavia porcellus* (Guinea Pig) L-ASNase but found issue with gathering ample Guinea Pig serum. Our research utilized *in silico* mapping to find the most recent common ancestor between guinea pigs and *Homo sapiens* (humans) and, using Ancestral Sequence Reconstruction, generated ancestral L-ASNases to determine which were closest in similarity to human L-ASNases while retaining comparable clinical-grade activity seen in existing bacterial L-ASNases. Recombinant L-ASNases were generated through the retaining of active sites of the ancestral L-ASNases, with the ancestral C-termini replaced with the human C-terminus. The recombinant enzymes were cloned into mammalian expression plasmids and transfected into two types of mammalian cells: Human Embryonic Kidney (HEK) Expi293F and Baby Hamster Kidney (BHK) cells. Early asparaginase assays measuring the production of aspartate and ammonia (asparaginase byproducts) demonstrated the success of the recombinant enzymes, but subsequent attempts to generate large amounts of the proteins were unsuccessful. Multiple attempts at generating stable cell lines for each L-ASNase of interest were also unsuccessful. The current stable cell line of BHK cells transfected with the Ancestral 104 recombinant (An104) plasmid shows promise in the generation of a stable cell line that produces the An104 L-ASNase, warranting the scale-up of the stable line to create a kinetic profile for the enzyme. Research into other ancestral recombinant L-ASNases that are closer in genetic similarity to humans is also warranted to determine the ideal candidate which retains clinical-grade asparaginase activity while increasing the similarity to the human asparaginase to reduce immunogenicity.

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Introduction

Background

L-asparaginase (L-ASNase) is a critical component of the chemotherapy armamentarium used to treat acute lymphoblastic leukemia (ALL). Precise disease risk stratification and intensification of multi-agent chemotherapy regimens have greatly improved the outcomes in pediatric ALL, with the 5-year event-free survival improving from 60% in the mid-1970s to now exceeding 90%. L-ASNase was first introduced into ALL regimens in 1978 and has played a significant role in improving these outcomes. Current L-asparaginases are bacterial in origin, derived from either *Escherichia coli* or *Erwinia chrysanthemi*, and hence are highly immunogenic due to ~30% identity shared with the human version of L-ASNase, resulting in reactions ranging from silent inactivation to severe anaphylaxis. Recent data has shown that discontinuation of L-ASNase results in poorer prognostic outcomes in ALL. Additionally, a significantly higher level of liver and pancreatic toxicity is seen in adults with L-ASNase, thus limiting its widespread use. Emerging data suggests that toxicity is related to the concurrent glutaminase activity seen in bacterial asparaginases. Thus, development of a less immunogenic asparaginase with reduced glutaminase activity is essential to overcome the major deficiencies of the current bacterial L-ASNase products. Enzymatically, L-ASNase is an amidohydrolase, which catalyzes the production of free L-aspartic acid from the non-essential amino acid L-asparagine, thereby

depleting the circulating pool of L-asparagine. ALL blast cells which lack asparagine synthase activity are dependent on the extrinsic supply of asparagine for protein synthesis and are thus extremely sensitive to L-asparaginase. The anti-tumor properties of L-ASNase were serendipitously first discovered in 1953 by J.G. Kidd who demonstrated regression in subcutaneous lymphomas in mice when treated with guinea pig serum. A decade later J. D. Broome confirmed that the anti- lymphoma effect was from L-ASNase in guinea pig serum. The difficulty in manufacturing adequate amounts of guinea pig L-ASNase in the pre-molecular biology era led to researchers pivoting to bacterial sources from L-ASNase production. Immunologic and non-immune mediated toxicity toxicities preclude up to 25% of patients from completing a full course of E. coli derived L-ASNase. Patients who have an allergic reaction to E. coli derived L-ASNase now receive crisantaspase, the L-ASNase native to E. chrysanthemi. There remains no L-ASNase substitute for the 10-30% of patients who have reactions to crisantaspase. The application of recombinant technology in L-ASNase production, however, provides an opportunity to overcome the current toxicity associated with bacterial asparaginases. Our team has pioneered the ancestral sequence reconstruction (ASR) approach for pharmaceutical protein drug discovery, *e.g.* humanized uricase, and enhanced blood coagulation factors VIII and IX, and von Willebrand factor. ASR utilizes extant sequences to predict ancient DNA and protein sequences, permitting higher-resolution mapping through comparisons of sequential phylogeny branches in contrast to other protein-drug design approaches such as ortholog scanning (*i.e.*, creation of interspecies hybrid proteins). This innovative ASR approach was used in the current study for L-ASNase drug discovery. We have generated Ancestral L-ASNase (An-L-ASNase) candidates possessing i) comparable ASNase-specific activity, ii) similar cytotoxic

activity against ALL cell lines, and iii) lower predicted immunogenicity risk compared to existing commercial ASNase products. This was achieved by examining the ancestral enzyme promiscuity and functional diversification along the human and guinea pig convergent lineage, highlighting important functional residues (Figure 1). The human guinea pig axis was chosen because despite sharing 70% sequence identity with human L-ASNase, L-ASNase derived from guinea pig possesses significantly favorable anti-leukemic, enzymatic properties and reduced glutaminase activity. Our goal is to further refine lead L-ASNase drug candidates by identifying the amino acid substitution(s) responsible for the enhanced activity and create a more humanized ancestral L-ASNase drug product.

Hypothesis

Reductionist screening approaches, including domain swapping and scanning residue substitution will enable the identification of next-generation humanized L-ASNase candidates with superior pharmaceutical properties. This can be achieved through designing and characterizing ancestral-human hybrid L-ASNases which retain ancestral enzyme active sites and incorporates the human C-terminal domain of unknown function.

Aims of Study

This project aims to generate L-ASNase constructs with ancestral active sites, which offer similar/improved activity to existing clinical-grade asparaginases currently used in

chemotherapy treatment for ALL, and human c-terminuses, which will lower the risk of immunogenic responses in patients. The first goal is the successful cloning of the constructs into a mammalian expression plasmid (pcDNA 3.4). The second goal is the creation of "stable" mammalian cells that have the cloned plasmids integrated into their genomes to create cell lines capable of producing the recombinant asparaginases. The third goal is to determine the kinetic parameters for each of the recombinant asparaginases to compare their activities to one another and to existing asparaginase products.

Methods and Materials

Ancestral-human hybrid L-ASNases and relevant controls were engineered and ordered through GenScript: Human *(Homo sapiens),* Guinea Pig (*Cavia porcellus),* Ancestral 85 (An85), Ancestral 93 (An93), and Ancestral 104 (An104). All of the constructs contained their respective active sites (as named above), an IL2 marker for exocytosis, and a 6x his tag for nickel chromatography purification, with the ancestral sequences containing a human C-terminus. These plasmids and a pcDNA 3.4 plasmid were digested with Xho1 and Not1 to isolate the inserts from the ordered plasmids, loaded onto a 1% agarose gel, ran through gel electrophoresis, and purified using the Omega Gel Extraction Kit following the manufacturer's instructions. The purified inserts were ligated into the cut pcDNA 3.4 plasmid using NEB T4 DNA ligation protocol and placed into the Thermocycler overnight to control the ligation temperatures. The ligations were transformed into bacterial cells, put in 200µl LB broth, plated onto ampicillin+ plates, then placed the plates

in the 37°C incubator overnight. Three colonies were selected and grown up in 5ml LB broth + 5µl ampicillin at 37°C overnight. Glycerol stocks were made from 500µl of each cultured media and 1ml glycerol, then froze at -80°C. The other 4.5ml of cultured media were purified using the QIAprep Spin Miniprep kit to extract the plasmid from the bacterial cells. Clones 1 and 2 for Human, Guinea Pig, An85, and An93 as well as clone 2 for An104 were sent to Plasmidsaurus for sequencing. After confirming the sequence accuracy of the ligated plasmids, Expi293F cells were grown up in F17 media at ~95% viability and a live cell concentration of ~3 x 10^6 cells/ml. The concentration of cells was brought up to ~1 x 10^7 cells/ml and 10 vials of 1ml of cells with 7.5% DMSO were frozen in liquid nitrogen. The remaining cells were kept at a concentration of ~3 x 10^6 cells/ml and maintained above 80% viability. The concentration of the cells was brought to ~200,000 cells/ml and 2ml was added to 5 wells of a 6-well plate, with the 6^{th} well containing 2ml F17 media (no cells). Human clone 1, Guinea Pig clone 1, and An104 clone 1 were transfected into three of the wells using Transit X2 and its respective procedure. The 6-well plate was placed on the shaking incubator at 37°C with 5% CO_2 and left to incubate for 48 hours. 1ml of each transfection was removed, spun down at 300 rcf for 5 minutes, and removed supernatant. Afterwards, the supernatants were frozen at -80°C. A crude enzymatic assay with Nessler's reagent was performed to verify the secretion and activity of the asparaginase recombinants, with Rylase and Asparlase as positive controls and the F17 media and naïve cell supernatants as negative controls.

The naive Expi293F cells were kept at a concentration of 3-5 x 10^6 cells/ml above 90% viability for ~20 days with splits on M/W/F, then spun down at 300rcf for 5 min and resuspended so that the concentration of cells was 1 x 10^7 cells/ml. Dimethyl sulfoxide (DMSO) was added to the

media with the cells to bring the DMSO concentration to 7.5%. The cells were aliquoted into 1 ml cryopreservation tubes and placed into a container with isopropyl alcohol in a -80°C freezer overnight to facilitate controlled rate freezing. The cells were placed into a container in liquid nitrogen after ~24 hours for storage over the holidays. Upon returning, one vial of frozen naive Expi293F cells was thawed on ice, spun down at 244 x g for 10 min at 4°C, then resuspended in 20ml F17 media in a 125ml Corning Erlenmeyer flask to bring the concentration to 5×10^5 cells/ml. The cells were then kept on the shaking incubator at 37°C 5% CO_2. The viability and concentration were measured to keep track of the cells' progress over the next few days following the thaw. After the cells had reached a concentration of 1×10^6 cells/ml above 90% viability, half of the volume of cells (10ml) was aliquoted and spun down at 300 rcf for 5 min before resuspension to bring the concentration to 2.5×10^5 cells/ml for transfection. The Transit X2 vial was thawed on ice and gently vortexed before using, the plasmids Human clone 1, Guinea Pig clone 1, An104 clone 2, An85 clone 1, and An93 clone 1 were thawed at room temperature, and the OptiMEM was warmed up in the 37°C water bath. 250µl OptiMEM, 2.5 µg plasmid, and 7.5µl Transit X2 were added to five tubes, with each tube containing one type of plasmid. The mixture sat at room temperature for 30 minutes. 2 ml of naive cells were added to each well of a 6-well plate, then one transfection mixture per well was added for five wells, with the sixth well remaining as naive cells. The 6-well plate was put on the shaking incubator 37°C 5% CO_2 for 24 hours. After 24 hours, the viability of the cells was checked and then the antibiotic selection agent Geneticin (G418) was added to each well to bring the G418 concentration to 400µg/ml, which is the selection dosage for obtaining stable cell lines. The 6-well plate was stored on the shaking incubator for ~96 hours due to a snowstorm that

prevented access to the lab for three days. The concentration and viability of the cells were checked following the storm. The transfected cells in wells were spun down and resuspended with 7ml F17 media at 400µg/ml G418 in 125ml Erlenmeyer flasks. All flasks were stored on the shaking incubator.

The top of the glycerol stocks Human clone 1 (Hum 1), Guinea Pig clone 1 (GP 1), and An104 clone 2 (An104 2) were scraped with wooden sticks and placed sticks in 50ml LB broth + 75µl ampicillin bacterial growth tubes, then placed in a 37°C shaking incubator for 12-16 hours growth. The plasmids from the inoculations were purified using the QIA Plasmid Plus Midi kit high-yield protocol. The process of inoculation and purification was repeated two more times over the next two days to gather ample DNA for a large-scale transfection. Digests of each plasmid (Hum 1, GP 1, An104 2) were made with 5µl 10X CutSmart Buffer, 1µl DNA (>300ng), 1µl Xho1 and 1µl Not1, and 42 µl water (50µl total digestion volume). The digests were placed in the 37°C incubator for 1 hour then were placed on a heating block at 56°C for 20 minutes to heat inactivated the enzymes. The digests were then stored in -20°C. Large scale transfections for Hum 1, GP 1, and An104 2 were made using 95 ml of a concentration of 3×10^5 cells/ml for each transfection. The three transfection mixtures composed of 10 ml OptiMEM, 300µl Transit X2, and 100µg of respective plasmid DNA, allowed to sit at room temperature for 30 minutes, and were then added to the 95ml of naive cells in the 500ml Erlenmeyer flasks and put on shaking incubator for 72 hours. 2 ml of each transfection were taken and added to a 6-well plate, with G418 added to bring the concentration to selection dosage (400µg/ml). The remaining transfection volumes were spun down and the supernatants were collected. The cells were then resuspended in 100ml F17 media. The collection was repeated after 48 hours.

Following the spin-down, the cells in the 500ml flasks were resuspended in 25ml F17 and G418 was added to bring the concentration to the selection dosage. After one week, the concentration of G418 in 6-well plate was reduced to maintenance dosage (200µg/ml G418). The proteins of interest from the transfection supernatants were purified via separate Ni-NTA 5ml Cytiva HisTrap HP columns using the AKTA. A binding buffer (500mM NaCl, 20mM Na_3PO_4, 20mM imidazole) and an elution buffer (500mM NaCl, 20mM Na_3PO_4, 500mM imidazole) were used. The Guinea pig and Human supernatants were eluted stepwise (5 column volumes binding buffer after the load then ~5-8 column volumes 100% elution buffer) and the An104 supernatant was purified with a gradient (gradient rose to 100% elution buffer over 15 minutes at flow rate of 2.5ml/minute and 2.5ml fraction volumes). The guinea pig and An104 elutions were concentrated using Amicon Ultra Centrifugal Filters (50kDa molecular weight cut-off) and were spun at 6000rcf for 5 minute intervals, then run on SDS-Page gels (210V for 30 min) to verify the existence of the asparaginases. The concentrated elutions were dialyzed using Slide-A-Lyzer Dialysis Cassettes sized 20,000 MWCO with a 0.5-3.0ml capacity. A Western Blot was run on the dialyzed and concentrated elutions as well as some bacterial purifications for An104 ancestral constructs using the mouse anti-His antibody as the primary antibody and Goat Anti-Mouse IgG1 (HRP) as the secondary antibody. After two weeks, transfection stables from the large flasks (40ml F17 at 200µg/ml G418) had few cells so they were spun down and resuspended in 5ml F17 at selection dosage, then were placed in a 6-well plate with 2.5ml of each transfection in two wells. Baby Hamster Kidney (BHK) cells were gathered from a split from another lab to attempt to create stables using an adherent cell platform. BHK cells were originally grown in 1X Dulbecco's Modified Eagle Medium (DMEM). Digested Human clone 1,

Guinea pig clone 1, and An104 clone 2 plasmids using the Not1 restriction enzyme to linearize the plasmid before transfection. Three 6-well plates were created, with one for each cell type that was transfected: suspension Expi293F cells, adherent Expi293F cells, and adherent BHK cells. Each 6-well plate contained a media-only well, a naive cell well (no transfection), a mock well (transfection reagents without any DNA), and the three plasmid constructs (Human, Guinea pig, An104) in the remaining wells. The suspension well plate was stored on the shaking incubator and the adherent well plates were stored on the shelf of the 37°C incubator for 72 hours. 1ml supernatants were collected from each well of the transfection well plates at the 72 hour and 120 hour marks. Nessler's reagent assays were run on the collections. A portion of the supernatants was dialyzed for further enzymatic analysis. SDS-page gels were run on all of the collections from the 72 and 120 hour marks. BHK cells were plated in 5 10cm petri dishes and grown in 1X DMEM until they reached ~95% confluency. 20µg of the Hum 1, GP 1, and An104 2 plasmids were digested with the SacII restriction enzyme. The transfection reagents (1.5ml OptiMEM, 45µl Transit X2) were added to four 15ml falcon tubes labeled Human, Guinea pig, An104, and Mock, then 20µg of each digested plasmid was added to the respective tubes, with no DNA added to the Mock tube. The mixtures sat for 30 minutes at room temperature to allow the formation of transfection complexes. The media was replaced in each dish with 14 ml 1x DMEM before the transfection. The transfection reagent complexes were added to the four respective dishes: Human, Guinea pig, An104, and Mock. No transfection reagents nor DNA were added to the Naive dish. All dishes were placed in the 37°C incubator. 1ml of supernatants was collected from each dish after 48 hours. Following the collection, the media was replaced

for each dish with 15 ml 1x DMEM, with the four transfected dishes containing a selection dose of G418.

Modified Nessler's Reagent Protocol

L-asparaginase activity was measured using a modified Nessler's reagent plate-based assay. Ammonia standards were prepared from ammonium chloride (NH_4Cl) in concentrations ranging from 0 to 64 µmoles. A 200 µL aliquot of each standard was added to the appropriate wells, and absorbance was measured at OD405 nm using a microplate reader.

For the experimental assay, each well contained 160 µL of 50 mmol L^{-1} Tris–HCl (pH 8.6), 0.1µM L-asparagine, and 0.1 mg/mL L-asparaginase, with the final volume adjusted to 336 µL using Milli-Q water. The reaction mixture was incubated statically at 37°C for 5 minutes, after which 16 µL of 1.5 mol L^{-1} TCA was added to stop the reaction.

Ammonium quantification was performed by transferring 35 µL from each reaction well to a fresh microplate containing 35 µL of Nessler's reagent and 280 µL of water. Following a 10-minute static incubation at room temperature, absorbance was measured at OD405 nm using a microplate reader, and NH_4^+ concentrations were determined using standard curve.

One unit (U) of L-asparaginase is the amount of enzyme which generates 1µmole of ammonia in 1 min at 37 °C and pH 8.6.

Asparaginase Colorimetric Activity Assay

L-asparaginase activity was assessed using the ABCAM colorimetric assay following the manufacturer's protocol. Briefly, aspartate standard solutions ranging from 0 to 10 nmol/well were prepared. L-asparaginase samples (10 nM) and asparaginase positive controls (5 µL) were loaded into wells, with volumes adjusted to 50 µL using assay buffer. Background control wells were also included. All standards, samples, and controls were analyzed in duplicate. A 50 µL volume of Reaction Mix was added to each well, followed by a 10-minute incubation. Kinetic measurements were recorded using a microplate reader at OD570 nm every 15 seconds for 60 minutes at 25°C, protected from light.

Activity of Asparaginase is calculated as: $\Delta A570nm = (A2 – A2BG) – (A1 – A1BG)$, where

A1 is the sample reading at time T1.

A1BG is the background control sample at time T1.

A2 is the sample reading at time T2.

A2BG is the background control sample at time T2.

Results and Discussion

Experimental Successes

The full plasmid sequencing of the cloned plasmids showed the successful cloning of all 5 constructs (Human, Guinea Pig, An85, An93, An104). The results indicated no errors present in the Human, Guinea Pig, and An104 clones.

Hum 1, GP 1, and An104 2 were all successfully transfected into Expi293F cells, where the cells incubated for 48 hours (in F17 media in 37°C and 5% CO_2) before the supernatant was removed and analyzed using a Nessler's Reagent assay that tests the presence of ammonia, a product of the asparaginase-catalyzed reaction. The assay revealed the success of the transfections as well as the presence of activity for the Guinea Pig and An104 enzymes. The assay showed that even without purification and concentration of the supernatants, the Guinea Pig and An104 asparaginases showed activity in the assay (Figure 3).

The large-scale transfections (95 ml in each flask for each construct) were able to make the largest amount of protein seen in the experiment. The two collections spaced at 72 hours and 120 hours from the initial transfection were pooled and purified to gather the maximum amount of protein. Through the repeated Ni-NTA purifications for the L-ASNases, it was determined that a stepwise elution of the protein was able to gather more of the protein than a gradient elution. This resulted in a slight loss of some protein for the An104 construct but ended up informing us of our future directions with purifications. The purifications collected were able to participate in enzymatic assays to determine the activity of the constructs.

Aspartate and ammonia-based assays were run for the elutions, where Guinea pig showed asparaginase activity in the aspartate and Nessler's reagent assay when compared to the An104 elution; however, the Western blot (Figure 4) revealed that Guinea pig was the only protein in a high enough concentration to be present on the transfer paper labeled with the anti-His tag antibody. This leads us to believe that the results of the asparaginase assays (Figures 5b and c) had less to deal with the activity difference between the proteins but rather the amount of each protein present. The concentrations of the proteins were still far from the clinical-grade asparaginase Rylase even when Rylase was diluted 1:3000 for the assay. When the dilutions were compared on a gel, only the Rylase band was present, suggesting again that the results of the assays were more likely to be indicative of protein concentration rather than enzymatic efficiency. Raw supernatants of the ongoing stable transfections revealed that the media that the cells were grown in (F17) contains both aspartate and ammonia, both of which make the signal from the media too high to be registered as a background for enzymatic analysis. Concentrations of the proteins were not established for these supernatants and therefore it is unclear what the comparisons show regarding the activity of the enzymes and the concentrations that they are operating at.

The third attempt at creating stable cells utilized the linearization of the plasmids as well as two cell lines (Expi293F and BHK) and two growth forms (suspension and adherent) to create three cell types for comparison (suspension Expi293F, adherent Expi293F, adherent BHK), each transfected with all three plasmids (Human, Guinea pig, An104). The plasmids were linearized

with the Not1 enzyme before transfection to maximize the likelihood of incorporation into the nuclear genome of the transfected cells. Without genome incorporation, daughter cells would not have the transfected gene and would not be able to produce the constructs. The suspension Expi293F and adherent BHK cell lines appeared to have been successfully transfected, which was seen when the cells were placed under multiple rounds of selection dosage of G418 and still had living cells in the respective wells. The selection terminated the cells where the transfected DNA was not incorporated into the nuclear genome, as the antibiotic resistance for G418 was included in the transfected DNA.

The current attempt at generating stable cell lines for each construct utilizes the BHK cell line alone with an adherent platform in 10cm petri dishes. The plasmids were linearized with the SacII restriction enzyme which targets a cut site outside of coding regions of the plasmid while retaining the complete insert as well as the poly-A tail and the antibiotic resistance region. The cells were plated and allowed to reach 95% confluency before transfection to ensure the maximum amount of cells that could be transfected. The cells were placed under selection 48 hours after transfection to give them ample time to incorporate the linearized, transfected DNA into their nuclear genome. After two weeks, the An104 stable line showed confluency in the 10cm petri dish and the cells were transferred to a T75 flask to give the cells more area to grow on.

Experimental Failures and Troubleshooting

When the full plasmids were sequenced, the An85 and An93 clones showed a large deletion (9 bp) in the region encoding the Interleukin-2 signal peptide. An85 and An93 were recloned from the initial plasmid sent by GenScript to yield the same result, revealing a defect in the creation of the An85 and An93 plasmids by GenScript. This was found to be due to an ordering error where the initial three amino acids of the protein were deleted from the order form before it was sent in, resulting in the 9 bp deletion from the DNA sequence and rendering the IL2 tag useless.

The first attempt at creating stable cell lines was ultimately unsuccessful. When put under multiple rounds of selection, few cells survived. It is possible that the short window in which they were allowed to incubate was not long enough for the cells to incorporate the transfected DNA into their genomes before being put under selection dosage of G418. Without genomic incorporation, the cells would not be able to pass the antibiotic resistance and construct DNA to their daughter cells. It is also possible that the suspension cells were scaled up from the 6-well plate to an Erlenmeyer flask too quickly, which may have affected the growth rate and health of the cells as suspension cells tend to grow best at a minimum of 5×10^5 cells/ml. This would mean that the scale up to the Erlemeyer flask diluted the cells to a concentration where the cell density was too low to promote ample growth, which could've caused the cells died out.

The large-scale transfections ultimately resulted in experimental failure, most likely due to the breakdown of the incubator. It is unclear when the breakdown occurred, but it is believed to have occurred sometime between 24 and 48 hours after the transfections took place. The lack of an optimal growth environment negatively impacted the health of the cells, which were already impacted from the transfection itself. The media collections showed that minimal protein was made from the flasks despite being given an optimal growth period and rebound time through the two collections spaced 72 hours and 120 hours from the initial transfection. This leads us to believe that a large number of the cells containing the DNA of interest died or were growth inhibited due to the conditions they were subjected to. The gels show the concentrated and dialyzed elutions from the Ni-NTA purifications for the Guinea pig and An104 proteins, which show that the respective asparaginases are present in the elutions but at a low amount.

The attempt at generating stable cells from the large-scale transfections ultimately failed in both environments (the 2ml in wells and the concentrated flasks). It is possible that the incubator breakdown prevented some cells from surviving long enough to incorporate the transfected DNA into their genome despite being given ample time to incorporate the plasmid. It is also possible that the chance of genomic incorporation was impacted due to the fact that the plasmids were not linearized prior to the transfections. The 2ml in the 6-well plate could've also been scaled up to the 125ml Erlenmeyer flasks too quickly like the previous stable generation attempt, resulting in a cell density too low to promote growth. For the other platform, it is possible that the four-fold concentrating of the cells in the 500ml flasks from

100ml to 25ml of media could've forced the cells to a density that was too high for growth, especially if there were a large number of dead cells from the incubator breakdown. Adding the selection dose of G418 could've exacerbated the problem, as even more dead cells would've been generated. A lower centrifugal speed was used to try and remove the large number of dead cells, where the living cells were denser than the dead cells and would remain at the bottom of the tube while the dead cells would float in the supernatant for removal, but it is possible that this lower speed did not draw all of the living cells to the bottom of the tube and some were discarded during the decanting of the supernatant. One other complication that was noticed was a "clumping" of the suspension cells, which made both cell counts and resuspension difficult. All cells were normally resuspended using stripette tubes, but both 1000µl and 200µl micropipettes were used to attempt to declump the cells by using a smaller opening for resuspension. Despite these attempts, the cells still clumped, which was problematic for cell counts as it resulted in larger numbers of cells being removed for cell counts that could've have had the plasmid construct incorporated into the genome but were removed from the media for the counts. The concentration issue also arose again, as after numerous spins and rounds of selection dosage of G418, the number of cells dwindled to a low concentration that prevented optimal cell growth. After roughly two weeks, the cells were concentrated down to 5ml and split between two wells in order to raise the concentration to promote growth. It is possible that during this process, the clumped cells were not distributed evenly between the wells or were lost in the transition. It is also possible that the clumped cells continued to be lost through the collection for cell counts, dwindling the already low number of stable cells in the wells.

The third attempt at creating stable cells utilized the linearization of the plasmids as well as two cell lines (Expi293F and BHK) and two growth forms (suspension and adherent) to create three cell types for comparison (suspension Expi293F, adherent Expi293F, adherent BHK), each transfected with all three plasmids (Human, Guinea pig, An104). The plasmids were linearized with the Not1 enzyme which, unbeknownst at the time, removed the 3' poly-A tail from the protein. This resulted in initial confusion as the suspension Expi293F and adherent BHK cell lines appeared to have been successfully transfected, which was seen when the cells were placed under selection dosage of G418. This finding was also supported by Nessler's reagent assay on supernatant collections from the media from all three cell lines at the 72 hour and 120 hour marks. The assays revealed no activity from any of the undialyzed supernatants as well as separate dialyzed supernatants for each cell line's Guinea pig, transfection, which were used for comparison of protein production between cell lines. After two weeks post-transfection, the error was caught, and the cells were discarded for the inability to make the proteins of interest despite successful transfection and stable cell formation.

The current cell line was successfully transfected and the three cell lines were placed under selection dose of G418 for one week and maintenance dose of G418 for the subsequent week. During that time, the human and guinea pig transfections died out. It is possible that they died due to failed incorporation of the transfected DNA into their nuclear genome.

Conclusion and Future Directions

L-ASNase is a critical component of the multi-drug approach to treating ALL, and existing clinical asparaginases are derived from bacterial sources such as E. coli or E. chrysanthemi. These bacterial asparaginases have high activity but trigger an immune response in ~30% of patients, rendering the treatment ineffective. Past research has shown that the Guinea pig asparaginase has high activity and a more similar protein makeup when compared to the human asparaginase ortholog. Our work with the creation of ancestral asparaginases *in silico* has allowed us to locate the most recent common ancestor between humans and Guinea pigs (An104) as well as multiple nodes between An104 and the human asparaginase that warrant investigation to find an asparaginase that displays comparable/improved activity to current clinical drugs as well as increased similarity to the human asparaginase to reduce immunogenicity. Our research has shown comparable activity with the ancestral proteins synthesized through the bacterial platform but had purification complications. Recombinant forms of the ancestral asparaginases An104, An93, and An85 were generated using domain swapping to retain the ancestral active sites and replacing the C-terminus with the human asparaginase C-terminus. The asparaginase inserts were cloned into mammalian expression plasmids and transfected into mammalian cells. The Guinea pig and An104 enzymes showed increased activity compared to the human L-ASNase but complications arose regarding the expression of increased quantities of the protein as well as the establishment of stable cell lines that could produce the proteins of interest. The two asparaginase assays that were used also were met with complications as the media that the cells were grown in contained the measured

substrates (aspartate and ammonia), rendering the background as too high for accurate measurements of the enzymatic activities between the raw supernatants of the cell lines. The initial linearization of the plasmids for stable cell acquisition also resulted in a complication, as the poly-A tail was separated from the protein and resulted in no production of the protein of interest. The future directions of the project include the establishment of stable cell lines for each protein so that more accurate enzymatic assays can be performed, as well as creating kinetic profiles for the recombinant ancestral asparaginase An104. The other two ancestral recombinants An85 and An93 will be reordered with the correct IL2 tags, cloned into mammalian expression plasmids, and transfected into cells to determine if there is similar/improved activity between them and An104. Stable cell lines of An85 and An93 will also be generated to provide ample protein for the use of the L-ASNases in a variety of enzymatic assays. Assuming confirmation of comparable/improved activity seen in one or more of the ancestral recombinants, the candidates will participate in cytotoxic assays to verify their impact on ALL cell lines. Glutaminase co-activity of the enzymes will also be established to determine potential risk complications to patients that come with higher glutaminase activity.

Reflection

This project expanded my knowledge of the biomedical research field and sparked an interest in pediatric oncology. It tested my problem-solving capabilities and taught me patience, that things can’t be rushed, and corners can’t be cut in the pursuit of novel drug development. It also taught me how the unexpected should be expected, and that adaptability is sometimes the

greatest strength one can have when working in research such as this. Working on this project taught me how to be thorough with my reasoning and with my experimental procedures, especially when dealing with topics that I might know less about than I anticipated. It has altered my career path to one which includes the pursuit of a research degree alongside my ongoing goal of a medical degree.

Figures

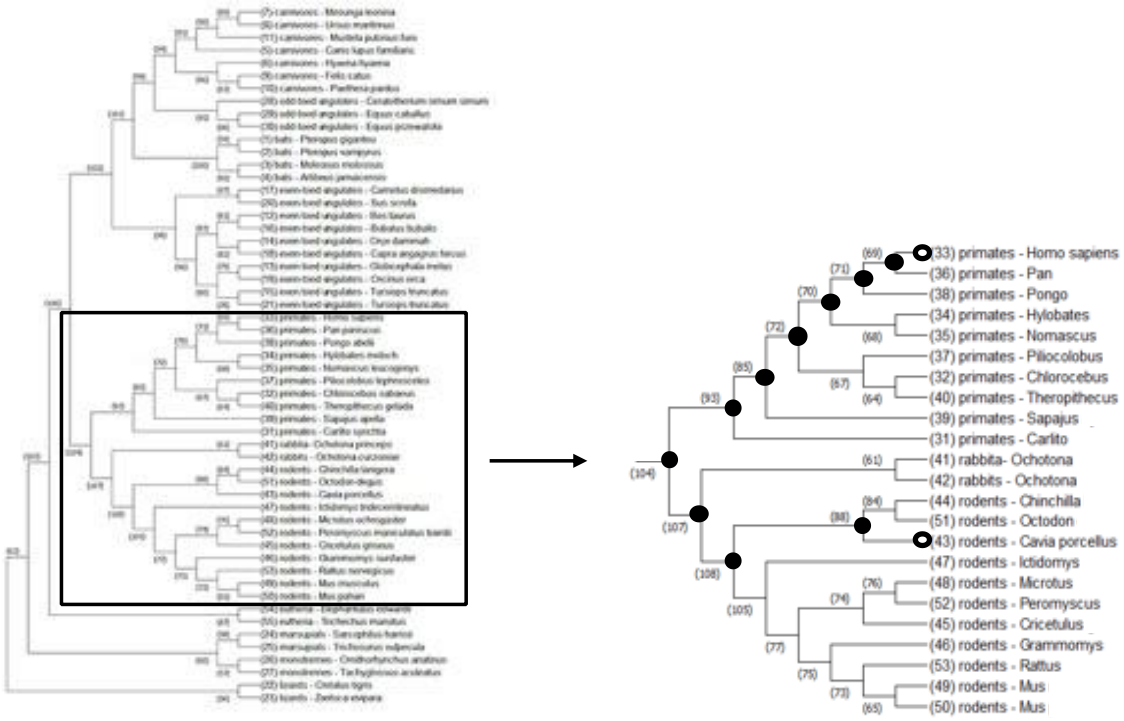

Figure 1: Phylogenetic tree inferred using MrBayes software. Ancestral nodes demarcated by black dots and numbered. *Homo sapiens* and *Cavia procellus* denoted by open circles.

Comparison of Ancestral L-asparaginase-Human Hybrid sequences to Human L-asparaginase		
L-Asparaginase	Identity to Human L-Asparaginase (%)	Amino-Acid Replacements
Human	100	0
An-69-h_c	99.13	5
An-71-h_c	98.95	6
An-70-h_c	98.78	7
An-72-h_c	98.95	10

An-85-h_c	97.91	12
An-88-h_c	89.70	59
An-93-h_c	94.94	29
An-104-h_c	94.07	34
An-107-h_c	93.72	36
An-108-h_c	93.19	39

Figure 2: Comparison of Ancestral L-ASNase-human hybrid sequences to human L-ASNase. "h_c" stands for "human c-terminus". Amino acid replacements refer to the number of amino acid differences from the human L-ASNase.

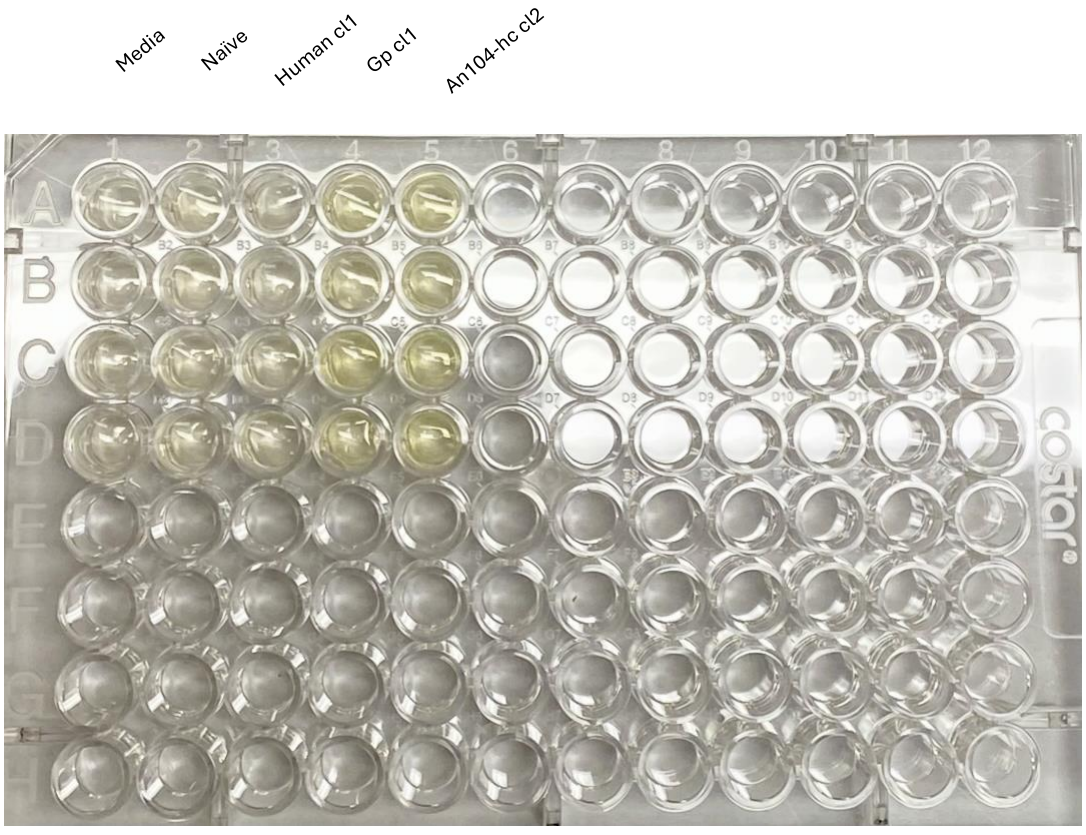

Figure 3: Nessler's reagent assay on primary transfections. All reactions were performed in quadruplicate. In order of columns: Media, Naïve, Human clone 1, Guinea pig clone 1, An104 clone 2. More yellow wells indicate a larger concentration of ammonia.

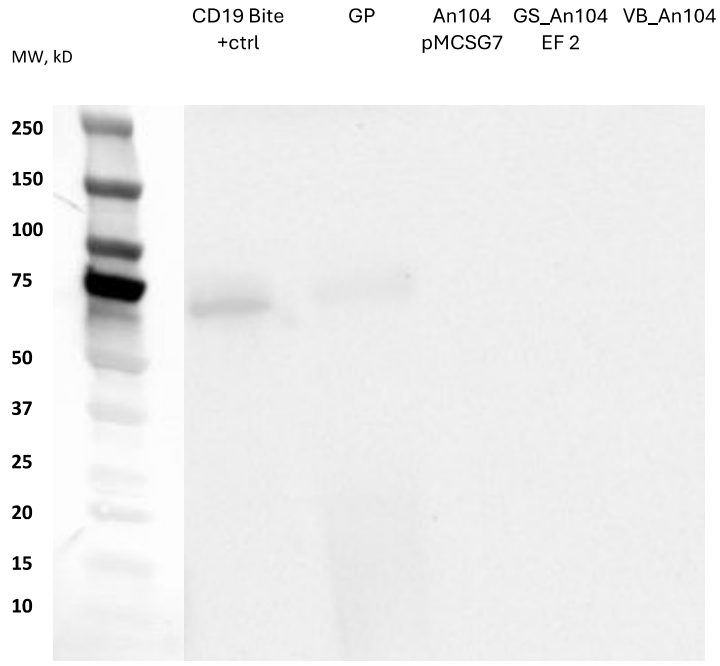

Figure 4: Western Blot ran on L-ASNase proteins after Ni-NTA purification. Proteins were purified, concentrated, and dialyzed. Mouse anti-His antibody used for the primary antibody and Goat Anti-Mouse IgG1 (HRP) used for the secondary antibody. CD19 BiTE used for positive control.

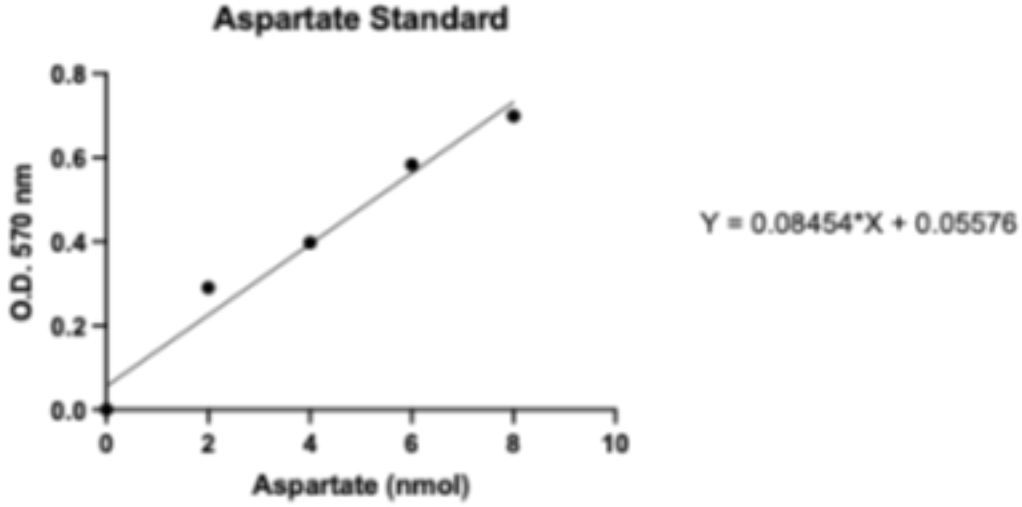

Figure 5a: Standard curve of aspartate.

Asparaginase activity (mU/mL) of purified proteins

Vb01_An104	0
Gs03_An104	0
An104 Human C Terminal	0
Guinea Pig	57.98

Figure 5b: Asparaginase activity of purified L-ASNases measured in mU/ml. 1 unit Asparaginase = the amount of asparaginase which generates 1.0 µmol of aspartate per minute at 25°C. Vb01_An104 and Gs03_An104 were synthesized on a bacterial platform and An104 Human C Terminal were synthesized on a mammalian platform.

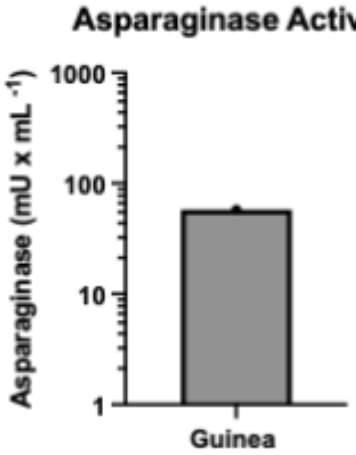

Figure 5c: Guinea L-ASNase activity. *E. coli* L-ASNase activity is listed as ≥ 100 mU/ml (from Australian and New Zealand Children's Haematology/Oncology Group)

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