Abstract: Cannabinoids, opiates, and venoms are used in the production of pharmaceuticals; unfortunately, these drugs can have adverse side effects or be costly to manufacture. With our project, we aim to produce biological systems that make the conjugates of these pharmaceuticals that have less negative side-effects, lack addictive properties and are inexpensive. To achieve this, three protein expression systems were designed: (1) An Agrobacterium-based system in tobacco plants for the synthesis of CBDA synthase, (2) Engineering the pGAPz alpha vector system to express mambalgin in Pichia pastoris, (3) and Manufacturing the constructs of Lethal Toxin-Neutralizing Factor LNTF-10 and LNTF-15 which are serum derivatives of the Didelphis virginiana (opossum) in the pSBC13 vector for assembly in Escherichia coli.
INTRODUCTION

Each year MIT holds a synthetic biology conference in Boston, this conference is called the International Genetic Engineering Machines(iGEM) competition. The iGEM competition is an annual, worldwide, synthetic biology event aimed at undergraduate university students, as well as high school and graduate students. Multidisciplinary teams work all summer long to build genetically engineered systems using standard biological parts called BioBricks. Teams over the summer strive to work inside and outside the lab, to create a project that will have a positive contribution to their communities and the world. The GSU-iGEM team decided this year to create a project aimed at producing safer, more cost-effective therapeutic alternatives for chronic pain, epilepsy, and snake bites.

The team decided to make the focus of the project the creation of therapeutic alternatives because it was observed that while individuals were suffering from various diseases are saved by the development and production of therapies and other health-related products created by the pharmaceutical industry. The factors driving human pathology continue to emerge, compound and diversify, and there has been a surprising lack of development to innovate and optimize remediation therapies (Light, BMJ). The team decided to construct protein systems to produce an anti-venom synthesized from peptides found in opossums, an analgesic derived from black mamba venom, and THC-free form of cannabidiol (CBD) oil.

Anti-venoms derived from snake venom are not always administered in a timely fashion due to the specificity of the treatment, and patients can have allergic reactions which can be just as deadly as the snake bite if the patient goes into anaphylactic shock. Antivenoms are also extremely costly to manufacture and are produced in small batches via extremely expensive procedures. To produce an alternative antivenom, it was decided to look for animals with natural
immunity for a solution. The opossum, while being the only North American marsupial, is also one of only a few animals on earth that are naturally able to neutralize multiple types of snake venom. This remarkable ability has been attributed to a series of peptides known as Lethal Toxin Neutralizing Factors (LTNF) (Lipps, 1999). These factors when tested in mice were found to completely neutralize multiple venoms as well as other toxins, such as ricin and botulism. Currently, the only way to obtain LTNF for experimentation is through a complicated and time-consuming protein purification using high-pressure chromatography on Opossum Serum (Lipps, 1999). Our plan is to take the genetic sequence for producing these peptides and clone it into E. coli cells for expression for generating a universal anti-venom and toxin neutralizer.

On the market, the most efficient pain treatments rely on opioid-based drugs which are highly addictive narcotics. An alternative pain medication needs to be developed, to develop a new therapy the team decided to look to snakes instead of away from them. Mambalgin is a potent analgesic protein found in the venom of the Black Mamba snake (Dendroaspis polylepis). Mambalgin-1, the version found here, is a 3-finger toxin consisting of 57 amino acid residues forming three loops around a core in the shape of a hand. This protein has been found to inhibit acid-sensing ion channels (ASICs) in the central and peripheral nervous systems of mice through intraplantar and intrathecal injections. The inhibition of ASICs – significant contributors to the pain pathway in both mice and humans – decreases the sensitivity of nociceptive neurons to the perception of pain. The potency of mambalgin has been compared to the drug morphine. Unlike morphine, however, mambalgin has not shown an increase in tolerance over time (Diochot et al., 2012). Because of its potency and non-addictive properties, the potential of mambalgin as a pain reducer in humans is enticing. Synthetically producing this protein in large quantities would
invalidate the need to harvest and extract directly from snakes – a dangerous and costly process.

Cannabidiolic-acid (CBDA) synthase is the enzyme that catalyzes the oxidative cyclization of cannabigerolic-acid(CBGA) into cannabidiol acid or CBDA(Sirikantaramas, 2004). Cannabidiol (CBD) is a non-psychotropic constituent of the fiber-type cannabis plant which can be found in CBD oil, and it is obtained from non-enzymatic decarboxylation of CBDA (Takeda et.al, 2012). It is important to produce the CBGA to CBDA pathway synthetically independent of cannabis plants to ensure the creation of CBD and not psychoactive tetrahydrocannabinol (THC). This is important because CBD oil can be used as an effective form of treatment for various disorders such as seizures, cancer, anxiety, post-traumatic stress disorder, and Crohn's disease. Since the CBDA synthase is 83.9% similar to THCA synthase in its 544-amino acid overlap, it should be possible to synthesize CBDA synthase in a similar fashion THCA synthase which has been synthesized multiple times (Sirikantaramas, 2004).

The shift in political climate coupled with the inability of patients to pay for costly drugs has effectively encouraged the team to invest time in creating three bacteria based protein systems to produce alternative Cannabinoids, opiates, and venoms. The first system developed was an Agrobacterium-based system in tobacco plants for the synthesis of CBDA synthase. The next protein production system was engineered in the pGAPz alpha vector system to express mambalgin in Pichia pastoris. The final system created was to manufacture the constructs of Lethal Toxin-Neutralizing Factor LNTF-10 and LNTF-15 which are serum derivatives of the Didelphis virginiana (opossum) in the pSBC13 vector for assembly in Escherichia coli. These biological systems would manufacture conjugates of these pharmaceuticals with less adverse side-effects, lack addictive properties, and are inexpensive.
EXPERIMENTAL PROCEDURES

To develop new drug therapies three protein expression systems were designed.

**CBDA synthase production system**

Cannabidiol (CBD) can be easily produced with cannabidiolic acid (CBDA) (Sirikantaramas, 2004). CBDA is catalyzed by an enzyme, which is called CBDA synthase, through oxidative cyclization. To synthesize the CBDA synthase the core enzyme in the CBD biosynthetic pathway the cDNA sequence for CBDA synthase was inserted into the pORE binary vector system. The plasmid is then transformed into Agrobacterium. The Agrobacterium containing the CBDA synthase is then used to transfect tobacco plant tissue with agrobacteria. The know transgenic tobacco plants produce CBDA synthase in their roots. The roots of these plants will then be introduced to CBDA synthase substrate, cannabigerolic acid, which will be catalyzed to form cannabidiolic acid. The Cannabidiolic acid will then be separated and heated at 120°C for 20min to form cannabidiol. Because of the difficulties with producing CBDA synthase, we decided to use the same procedure to produce Horseradish Peroxidase (HRP) in the roots of transgenic tobacco as a proof of concept. One of the benefits of this is the bioluminescence that is produced when it catalyzes its substrate and allows us to prove that the same process could work for the production of CBDA synthase.

**Synthesis of Mambalgin protein in Pichia pastoris**

Two biological systems were developed to produce the analgesic mambalgin-1 protein. To create a fast growth and easy cultivation system for the mambalgin construct a production system was developed in the organism *Escherichia coli*. For E.coli system, it was necessary to the first codon optimized the Mambalgin-1 sequence (BBa_k1110003) for *Escherichia coli* using the IDT codon optimization tool. Restriction sites were then removed from the coding sequence
that wasn't iGEM incompatible. An RFC 10 prefix and suffix were added, along with a start codon at the beginning of the sequence. Also, included in the construct was a prokaryotic RBS (BBa_j61101), IPTG-inducible promoter (BBa_R0011), and Myc and 6xHis epitopes. This fusion protein was designed specifically for easy expression in *E. coli*.

For the design of the Pichia Pastorsis system, the coding sequence for mambalgin was taken, the TAG stop codon removed, and added RFC 25 prefix and suffix in Snapgene software to visualize. RFC 25 was used because we were anticipating the utilization of this part in creating fusion proteins. Additional nucleotides were added before the bio brick prefix and after the bio brick suffix to enable more efficient restriction enzyme cutting at EcoRI and PstI sites. The mambalgin construct was then ordered from IDT and ligated into the pSB1C3 backbone. Using the pGAPza expression system provided by ThermoFisher Scientific, the GSU team has been working to express the protein in *Pichia Pastoris*. The pGAPza vector includes the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter, an alpha secretory signal peptide, and Myc and 6x His epitopes. The MCS, between the alpha secretion signal and epitopes, consists of restriction sites which will enable insertion of mambalgin. In-frame insertion into the MCS of pGAPza will be possible after using PCR to modify the construct - removing the RFC 25 prefix and suffix and adding restriction sites and nucleotides.

*Creation of Lethal Toxin-Neutralizing Factor (LTNF)*

For the design of LTNF, the initial step was to codon optimized the LTNF-10 (Bba_k1915001) and LTNF-15 (Bba_k1915003) for *Escherichia coli* using the IDT codon optimization tool. Restriction sites were then removed from the coding sequence that was not iGEM compatible. An RFC 10 prefix and suffix added, along with a start codon at the beginning of the sequence. Also, included in the construct was a prokaryotic RBS (BBa_J61101), IPTG-
inducible promoter (BBa_R0011), an HRV3C Protease Site and Myc and 6xHis epitopes. This protein was designed specifically for expression in E. coli.

RESULTS

**CBDA synthase part**

The part created for CBDAs included a promoter. This part is designed for constitutive expression of CBDA synthase in agrobacteria. This part is a composite of the J61002 promoter construct and our CBDAs coding sequence.

**Mambalgin part**

Expressed and characterized the mambalgin protein in *E. coli*. After inducing with IPTG overnight, the cell cultures were centrifuged and disrupted with a French press. Mambalgin was isolated using affinity chromatography, utilizing the 6x His tag in the construct. An SDS-PAGE was done, followed by a Ponceau S. and Coomassie stain to visualize the proteins. A band was indicating a protein of ~9 kDa – the size of the mambalgin for *E.coli* construct– was seen in the eluate fraction. A western blot was performed using an anti-His primary antibody. This year the iGem team repeated this process and successfully utilized the Myc tag to further characterize the mambalgin protein. Protein expression was also optimized this year by varying induction time as well as altering concentrations of IPTG.

**LTNF constructs**

LTNF-10 (Lethal Toxin-Neutralizing Factor) is a ten residue peptide that has been shown to neutralize toxins from snakes, scorpions, and bacteria. LTNF peptides were first isolated from opossum (*Didelphis virginiana*) serum. This part contains the LTNF10 peptide as well as C-term Myc and 6xHis tags that can be used for purification and then later removed using the HRV (Human Rhinovirus) protease.
Created the inducible lac promoter+LTNF-10 (Part: BBa_K1915003). This part is designed for inducible expression of LTNF-10 in bacteria. This part includes an IPTG inducible promoter, RBS J61101, and the LTNF15 peptide coding sequence.

LTNF-15 (Lethal Toxin-Neutralizing Factor) is a 15 residue peptide. This part contains the LTNF-15 peptide as well as C-term Myc and 6xHis tags that can be used for purification and then later removed using the HRV (Human Rhinovirus) protease.

Created the Inducible Lac promoter+LTNF-15. This part is designed for inducible expression of LTNF-15 in bacteria. This part includes an IPTG inducible promoter, RBS J61101, and the LTNF15 peptide coding sequence.

Proof of Concept: HRP in pORE

Horseradish peroxidase was inserted into the pORE vector and transformed into Agrobacterium tumefaciens. The Agrobacterium tumefaciens was used to infect tobacco plants to induce expression of hairy roots. To detect the horseradish peroxidase in the newly formed hairy roots, a chemical assay was performed. Into 5mL of deionized water ($dH_2O$) a piece of transformed hairy root along with 5mL of the chemical 3,3',5,5'-Tetramethylbenzidine (TMB) was placed into a 15mL microcentrifuge tube. The horseradish peroxidase turned a pale blue color in the solution.

FUTURE PROSPECT

Through synthetic biology, the team created five new parts, demonstrated expression of two of those parts, characterized the mambalgin protein, and transformed the tobacco plants with an HRP reporter construct as a proof of concept for our expression of CBDAS, then detected expression of our reporter under real-world conditions. For each protein system, the research is
still ongoing in order produce to biological systems that will perform as factories in the hope of providing a production system for inexpensive pharmaceutical derivatives.

To further develop the protein expression system for the Mambalgin-1 protein, the next steps include testing the analgesic properties in an animal model. To produce this model, the team has partnered with the Neuroscience Institute at Georgia State University to create An Institutional Animal Care, and Use Committee (IACUC) approved the experimental protocol.

To create THC free CBD oil, it was necessary to design a proof of concept. The plan laid out to prove the CBDA concept worked when the HRP enzyme in the transgenic tobacco roots interacted with the TMB substrate the solution turned blue! Thus, we know it is possible to insert CBDA synthase into the pORE vector and transform the recombinant plasmid into Agrobacterium to create tobacco plants that contain CBDA synthase. The presence of CBDAS will be confirmed with the use of mass spectrometry after purification from the aqueous suspension media that plants are grown in. Cannabigerol acid – the substrate for CBDAS – can then be added to produce cannabidiolic acid. The addition of heat to cannabidiolic acid will create cannabidiol. Functional testing in mammalian systems will commence after the acquisition of cannabidiol. Due to the lack of proper facilities in the iGEM lab for this type of chemical synthesis, it is necessary to collaborate with a chemistry lab.

The LTNF constructs require further characterization of the peptide to ensure that construct can indeed work as a universal antivenom. To do this, we will be performing a bioassay to determine the reactivity of the LTNF peptide to our mambalgin protein, an element of the mambalgin snake venom. NMR spectroscopy will be used to characterize the LTNF protein further.

In previous years, the progress of our project has been impeded by the obstacles related to
growing and maintaining healthy plants. After having entire crops die year after year, it was necessary to create a reliable system. This year we developed a relatively automatic care system that has proven to be both efficient and fruitful in maintaining plant growth. The automated homemade hydroponic growth system, nicknamed the Bulbasaur Nursery, has saved team time and money, while simultaneously allowing for plants to become a leading chassis organism in the lab. The next step for our nursery is to build an aquaponics system for the growth of more sterile tobacco plants.

REFERENCES


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