Engineering Yeast for Production of Semisynthetic Paclitaxel for Cancer Therapy

Background: Cancer is among the leading causes of deaths worldwide with approximately 38% of men and women diagnosed with cancer at some point in their lifetime.[1] With the rising cancer epidemic and the need for cheaper and more accessible drugs for people in developing countries, it is crucial to find new ways to develop pharmaceuticals. One sustainable method is engineering metabolic pathways of microorganisms such as yeast (*Saccharomyces cerevisiae*) or *Escherichia coli* to produce the precursor of a drug. One of most successful applications of this technique was achieved in Dr. Jay Keasling's lab at the Joint BioEnergy Institute by producing the precursor of the antimalarial drug artemisinin, reducing the cost 30-60% and ensuring a continuous supply.[2,3]

Engineering microorganisms to produce pharmaceutical products more efficiently can be applied to cancer drug development. Production of terpenoid and polyketide by engineered microorganisms would be particularly beneficial as small amounts of the molecules are produced via natural pathways and yields vary widely based on environmental and epigenetic factors.[4] Paclitaxel (brand name Taxol) is one of the most successful cancer drugs, and was first listed on the World Health Organization Model List of Essential Medicines in 2013. However, there was concern regarding the high cost of the drug[5], as it initially required the bark of six 100-year-old Pacific yew trees to treat a single patient with breast cancer.[6] Although alternate methods have been developed to extract paclitaxel from needles of the European yew, synthetic biology tools can be used as a more sustainable alternative. Since paclitaxel is a highly decorated diterpenoid (**Fig 1**), its complicated structure makes it a good fit to be engineered from yeast due to the highly versatile DNA transformation system and well-defined genetic system of yeast.

The objective of this project is to engineer a yeast strain capable of synthesizing paclitaxel which can be later optimized for commercial production. This will have an essential impact by reducing the cost of a crucial anticancer drug and providing valuable insight into the pathways required for the production of terpenoid and polyketide natural products from yeast. This project will focus on engineering taxadiene biosynthesis in yeast by utilizing glucose as the hydrocarbon source, studying and identifying cytochrome P450 oxygenation reactions in the pathway, and integrating these components to produce paclitaxel (**Fig 1**).

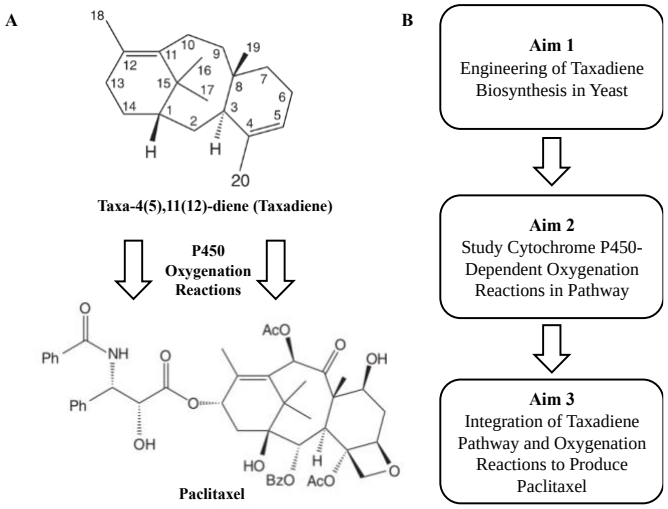

Figure 1. A: Taxadiene intermediate structure catalyzed via various cytochrome P450 oxygenation reactions to produce paclitaxel.[7] B: Identified route for production of paclitaxel.

Aim 1: Engineering of Taxadiene Biosynthesis in Yeast

Taxadiene (taxa-4(5),11(12)-diene) biosynthesis in yeast is crucial to the production of paclitaxel but the levels of various precursors these organisms produce naturally are insufficient to make the process feasible. The diterpenoid precursor for taxadiene, geranylgeranyl diphosphate (GGDP), is necessary for a heterologous taxoid biosynthetic pathway but is produced in insufficient quantities in yeast due to competition for steroid synthesis with farnesyl diphosphate. I will introduce heterologous genes from the *Sulfolobus acidocaldarius* GGDP synthase instead of the native GGDP synthase from *Taxus* for improved production of GGDP

and taxadiene as there is no competition for steroid synthesis.[4] Introduction of genes from *S. acidocaldarius* also results in substantial production of geranylgeraniol, further increasing taxadiene yields. Yeast will be transformed by the lithium acetate method[8] on SC minimal medium agar plates via CRISPR/Cas9 and select plasmids (pVV200 (tryptophane), pVV214 (uracil), pRS313 (histidine) and pRS315 (leucine)). Yeast will be cultivated for 48 hours with glucose as the carbon source, lyophilized, extracted with pentane, and analyzed by GC-MS.

Aim 2: Study Cytochrome P450-Dependent Oxygenation Reactions in Pathway

The oxygenation steps in the biosynthesis of paclitaxel have yet to be fully studied and identified. After taxadiene biosynthesis, oxidative modification of the olefin and the elaboration of side chains are needed to transform taxadiene into paclitaxel. After the taxadiene skeleton is formed, the olefin must be modified by several P450-mediated oxygenations and coenzyme A dependent acylations. Candidate genes for all but one of the seven steps after taxadiene synthesis are postulated, but the entire pathway has yet to be confirmed.[9] Uracil-specific-excision-reagent (USER) cloning will be utilized for site-directed mutagenesis of the identified genes and cytochrome P450s and USER primers will be designed using the online AMUSER tool. All intermediates will be characterized by GC/LC-MS.

Aim 3: Integration of Taxadiene Pathway and Oxygenation Reactions to Produce Paclitaxel

Once the taxadiene pathway and oxygenation reactions are identified and characterized, the pathways will be integrated via the lithium acetate method, CRISPR/Cas9, and site-directed mutagenesis to produce paclitaxel. Glucose will be used as the starter carbon source in yeast and will follow the native mevalonate pathway. The established genes to produce GGDP synthase will be introduced and the previously identified taxadiene synthase gene that has been codon optimized for improved yeast expression[4] will be incorporated to produce taxadiene. Then, the pathway developed with the cytochrome P450 oxygenation reactions will be introduced to produce paclitaxel. All intermediates will be evaluated using GC-MS. It is possible that some proteins synthesized in the complete pathway are insoluble or inactive in yeast, thus similar proteins will be determined or engineered to be active.

Intellectual Merit: My knowledge from chemical engineering coursework and research with developing cell cultures, DNA analysis, and molecular modification of chemical structures gives me an essential, well-rounded training for fulfilling this project. This project will be the first time a polyketide synthase (PKS)-terpene hybrid has been produced in yeast and will mark an imperative step in the industrial production of PKSs and thus, in the field of synthetic biology. I will collaborate with members of the Joint BioEnergy Institute to learn the genetic technique of integrating genes using CRISPR/Cas9 and use my knowledge of DNA sequencing and GC to confirm the genes responsible for paclitaxel and determine the developed molecules at each step.

Broader Impacts: Developing pharmaceuticals from microorganisms is an efficient and cost-effective way to produce the same high-quality compounds obtained from natural products. By engineering yeast to produce paclitaxel, a lower-cost, more sustainable drug could be developed for people suffering from lung, breast, or ovarian cancers who would otherwise not have access to the medicine. If successful, this project will provide a framework for synthesizing other PKS-terpene hybrid chemicals and pharmaceuticals from microorganisms.

References: 1. NIH, NCI. 2018. https://www.cancer.gov/about-cancer/understanding/statistics 2. Hale et al., Am Soc Trop Med. 2007. DOI: 10.4269/ajtmh.2007.77.198. 3. Ro et al., Nature. 2006. DOI: 10.1038/nature04640. 4. Engels et al., Met Eng. 2008. DOI: 10.1016/j.ymben.2008.03.001. 5. Mendis. WHO Model List of Essential Medicines. 2011. https://www.who.int/selection_medicines/committees/expert/18/applications/Mendis.pdf?ua=1 6. Horwitz, SB. Nature. 1994. DOI: 10.1038/367593a0 7. Chang et al., Nature. 2006. DOI: 10.1038/nchembio836. 8. Kaiser et al., Cold Spring Harbor Laboratory Press. 1994. ISBN: 0-87969-451-9. 9. Jennewein et al., PNAS. 2004. DOI: 10.1073/pnas.0403009101.