Developments in engineering the herpes simplex virus thymidine kinase for enzyme prodrug therapy using bacterial systems

By

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A dissertation submitted to Johns Hopkins University in conformity with the requires for the degree of Doctor of Philosophy

Baltimore, Maryland
September 2016

Abstract

The continuous search for better cancer therapies has fueled investigations into a diverse array of approaches aimed at treating the disease. An ongoing challenge in this field of research is the development of highly effective treatments that are also highly discriminating towards cancer. Early treatments used small molecule drugs targeting metabolic systems to exploit the higher metabolic rates of cancer cells for specific toxicity. In efforts to improve specificity, some of these small molecule drugs were later converted to prodrugs that could be activated by specific enzymes and utilized in gene directed enzyme-prodrug therapy (GDEPT). The herpes simplex virus thymidine kinase (HSV-TK) is one such enzyme that is used in GDEPT. Its promiscuity towards phosphorylating nucleoside analog prodrugs has led a strong drive to implement it in GDEPT-based cancer therapies. Efforts to engineer HSV-TK towards selective activity against prodrugs have proven to be moderately successful.

Here, we describe progress that has been made in advancing HSV-TK's utility in GDEPT. In order to produce selective prodrug activation in cancer cells, we used directed evolution to engineer HSV-TK into a protein switch that can be activated in the presence of the C-terminal transactivation domain (C-TAD) of the cancer-specific marker, hypoxia inducible factor 1α (HIF1-α). The CH1 domain of the p300 protein is capable of binding to the C-TAD and was inserted into the amino acid backbone of HSV-TK to sensitize the enzyme to the presence of the cancer marker. The protein switch, dubbed TICKLE (Trigger-Induced Cell-Killing Lethal Enzyme),

conferred a 4-fold increase in toxicity towards bacterial cells from azidothymidine (AZT) prodrug in the presence of C-TAD compared to the toxicity in the absence of C-TAD. The studies suggested that the switch was capable of phosphorylating AZT as well as thymidine (dT); thus, other nucleoside analogs could also act as substrates and increase TICKLE's prospects for utilization in various applications such as cancer therapy and as a molecular reporter gene. In addition, our attempts to address challenges in selecting for kinase activity in protein switch variants led to the development of a new bacterial positive selection for nucleoside kinases. The new selection overcomes toxicity issues present in previous selections and removes limitations that could enable the discovery previously undiscovered high activity variants of HSV-TK. The advances made in HSV-TK engineering in this work expand our knowledge of protein switch development and manipulation of bacteria for kinase selection. The progress can be directly applied to creating better enzymatic switches and HSV-TK variants.

Advisor: Marc Ostermeier, Professor of Chemical and Biomolecular Engineering, Johns Hopkins University

Readers: Phillip Cole, Professor of Pharmacology, Johns Hopkins Medical Institutions

James Eshleman, Professor of Pathology, Johns Hopkins Medical Institutions

Acknowledgements

First and foremost, I would like to express my endless gratitude to my parents, Lata and Yogesh Shelat. They emigrated from India with the sole purpose of providing a better education for their son. Their unrelenting love, support and encouragement have always been resounding throughout my life. A fitting thanks is difficult to convey within the limitations of words.

My friends have provided the perfect diversion from research. Particularly, Barrett Steinberg, who I thank for the homebrews, scientific discussions, music and companionship. I am also grateful to my friends Samuel Rosario and Amrita Gupta for always making themselves available whenever I needed their time (or needed to be distracted). Of course, this doctorate couldn't have been completed without Manan Jani, Ketan Rathod and Adithya Sundar. I have known each of them for two decades now and have grown to rely on their brotherly camaraderie, candid criticisms and unfailing loyalty through the years.

I must thank the members of the Ostermeier lab, starting with Dr. Marc Ostermeier. He allowed me to cultivate and pursue my own ideas, regardless of their merits. While many failed, the few that worked furthered my confidence in my own creativity. Tina Xiong, Courtney Gonzalez, and Tiana Warren have provided me invaluable assistance in lab, I couldn't ask for better colleagues and friends. I also want to express my gratitude to my committee members for their annual feedback and for reviewing this work. A final thanks to Dr. C. Allen Bush, who allowed a bright-eyed freshman into his lab and introduced him to scientific research. It serves as a testament to the importance of generosity and is an example I hope to live by.

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Chapter 1: Introduction & Background

Cancer & Treatments

Cancer is the second leading cause of death in the country and is responsible for 1 out of every 4 deaths. From the 1930s to the 1990s, the prevalence of the major forms of cancer stayed stagnant in the US population, except for lung and bronchus cancer, which displayed a staggering rise in incidence due to the tobacco epidemic (1). However, the mortality rates for most forms of cancer have seen a decline over the past two decades; this decline is thought have been fueled by a rise in awareness of external factors, such as tobacco consumption, that stimulate carcinogenesis and advances in cancer detection methods (2, 3).

While there is no specific cause of all cancers, all cancers are a result of genetic mutations that lead to disregulated growth and cell division. Carcinogenesis, the conception of cancer, is generally thought to occur through mutations in genes responsible for controlling cell division and apoptosis (4). The genes contributing to the development of cancer are classified as oncogenes, tumor-suppressor genes or genome stability genes. Oncogenes are responsible for promoting cell division and promote carcinogenesis upon up-regulation via activating genomic mutations. Tumor-suppressor genes are tasked with controlling the apoptotic response along with cell division, and the down-regulation of these genes through inactivating mutations contributes to carcinogenicity. Stability genes are utilized in preserving genomic integrity and correcting mutations; alterations in these genes cause a loss of genome editing and accelerate the development of carcinogenic mutations in

oncogenes and tumor-suppressor genes. Cells typically require multiple mutations at different genomic loci to overcome cells' genome correcting and apoptotic mechanisms (4).

Cancer cell proliferation can be aided by mutations in growth factors that lead to self-sustaining growth signals, lack of sensitivity towards anti-growth signals and sustained angiogenesis. The defective genes in the cancer cells also encourage more mutations in the genome, which can lead to metastasis. Limitless replication, apoptotic resistance, self-sufficient growth signals, lack of sensitivity to anti-growth signals, sustained angiogenesis and metastasis are known as the six "hallmarks of cancer" (5). These hallmarks also provide a way for the immune system and drugs to distinguish between regular cells and cancer cells.

Currently, chemotherapy is the most prevalent non-surgical treatment for cancer. The rapid proliferation of cancer cells is a characteristic that is partially facilitated by the cells' higher-than-normal metabolic rate; chemotherapeutic drugs have exploited this high metabolism to produce greater cytotoxicity towards cancer cells over normal cells. These drugs kill cells by actively inhibiting metabolic systems, such as DNA synthesis. Drugging metabolic systems results in cells with higher metabolic rates experiencing greater sensitivity towards the drugs than normal cells (8). However, normal cells also possess the metabolic capacity to process these drugs and are damaged when the drug metabolites interfere with cellular machinery. This damage is a significant contributing factor in the side effects that patients experience when undergoing chemotherapy (9). These intense

side effects have led to a renewed focus on developing targeted therapies that mitigate the amount of harm done to normal cells during treatment.

A plethora of approaches have been undertaken to increase specificity of cancer treatments towards cancer cells. The genotypic differences between cancer and normal cells allow the immune system to recognize cancer cells (7). However, cancer cells generate tumor microenvironments that help them evade immunosurveillance by expressing immunosuppressive proteins such as PD-1 (10). Recent advances in immuno-oncology have provided inhibitors to these immunosuppressive proteins to boost the immune response against tumors. In addition, many proteins on the cancer cell surface are tumor-specific antigens that can be utilized to increase the target-specificity of anti-cancer drugs. Attempts have been made to increase cancer-specific drugging by engineering nanoparticles with tumor-specific antigen-binding capacity to deliver the drugs (11). However, these particles can be absorbed by the liver and generate hepatoxicity.

Prodrug Therapy & Enzyme-prodrug Therapy

A novel approach to boosting target-specificity in cancer treatments is prodrug therapy. Prodrug therapy has been utilized for over 50 years to treat autoimmune diseases, cancer and viral infections (13). Conceptually, the treatment utilizes a drug that has been "inactivated" through the addition of a chemical moiety that renders it non-toxic. Once target cells have internalized the prodrug, target-specific factors remove the chemical moiety. In targeting cancer cells, these factors may be conditions such as hypoxia, which is prevalently found in tumors, or proteins that are found to be highly upregulated in cancerous tissue, such as

proteases (12). Since cancer cells share similar gene profiles as normal cells, it is difficult to design prodrugs that are capable of being activated exclusively in cancer cells. The last two decades have seen alterations and developments to prodrug therapy in order to overcome this issue.

Advances in gene delivery using viral and nanoparticle vectors now allow transgenes to be preferentially delivered to target cell populations. This is traditionally achieved by coupling antibodies capable of binding to tumor-specific antigens to delivery vectors or by manipulating the charge of the nanoparticles to increase their affinity towards certain cell populations (14). Once the vector reaches its target, the cell internalizes the vector's contents through endocytosis or membrane fusion (15, 16). The ability to successfully delivery transgenes to cells provides an alternative approach to classical prodrug therapy where exogenous, non-human genes capable of activating prodrugs delivered into targeted cells before prodrug administration. This method is known as gene-directed enzyme-prodrug therapy (GDEPT) (Fig. 1.1). A similar approach utilizes antibodies conjugated with an exogenous enzyme instead of viral or nanoparticles vectors housing a corresponding gene to the conjugated enzyme. This treatment approach is known as antibody-directed enzyme-prodrug therapy (ADEPT). The antibody in ADEPT targets tumor markers on the cell surface and the conjugated enzyme is capable of activating prodrugs that are delivered to the area.

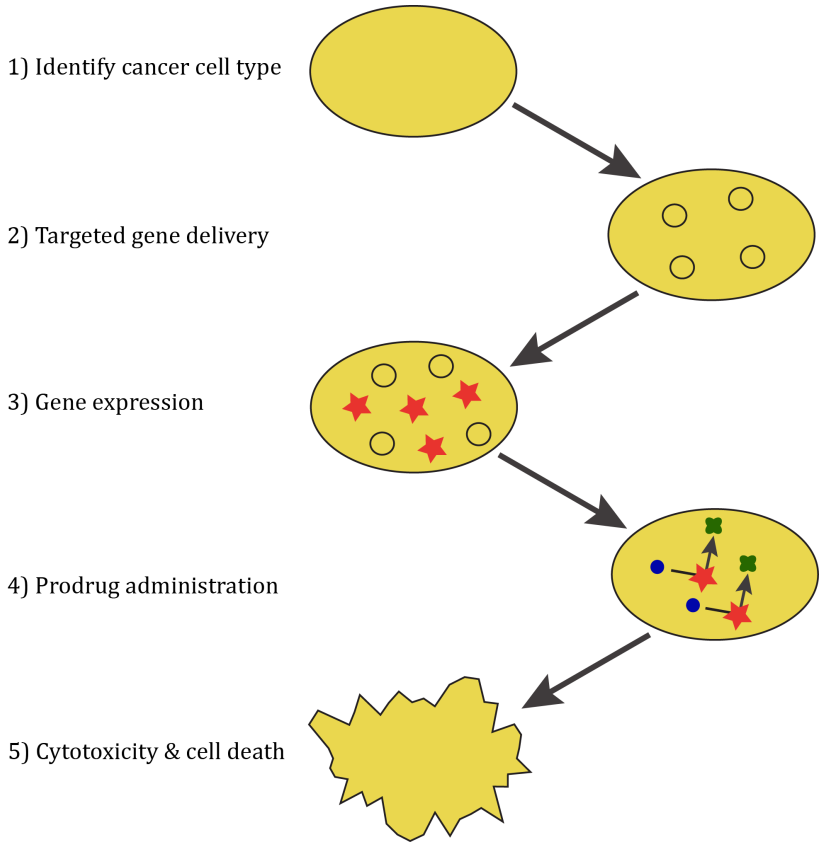

Figure 1.1: GDEPT Overview

The location of the tumor and cancer cell type is first identified to select best prodrug for treatment. After identification, a suicide gene is delivered to these cancer cells in a target-specific manner. After the gene is expressed within the cells, the prodrug is administered and cells harboring the suicide enzyme are killed when it metabolizes the prodrug into its toxic form.

By delivering exogenous genes and enzymes to targeted cells before administrating the corresponding prodrug, GDEPT and ADEPT are theoretically able to bypass multiple obstacles which plague classical cancer prodrug therapy. First, the task of finding suitable enzymatic targets in cancer cells that are not highly expressed in normal cells to activate prodrugs is removed. GDEPT and ADEPT can utilize any non-human enzyme as long as the prodrug is exclusively activated by it

and not by other proteins in the body. Second, the non-specific toxicity produced through the activation of the prodrug in normal cells is greatly reduced. With the ability to use prodrugs that are exclusively activated by specific non-human enzymes, non-specific activity is dampened. Third, with greater target specificity for prodrug activation, larger doses of the prodrug can be used and tumors experience greater regional toxicity.

However, from a practical standpoint, GDEPT and ADEPT face several challenges. First, gene and antibody delivery is an inefficient process. Delivery vehicles like nanoparticles interact with the mononuclear phagocytosis system (MPS) and tend to accumulate in liver and spleen instead of the targeted cell population (17). Second, both GDEPT and ADEPT can be potentially toxic. Using viral vectors can instigate an immune response in the body while the accumulation of nanoparticles in the liver and spleen results in systemic toxicity. Third, most gene and antibody delivery mechanisms are not extremely target-specific. Nanoparticles require tremendous size, structure and composition optimization to be effective yet the resulting target specificity is highly conditional on bodily responses to administration (19). Delivery through attenuated viral particles can also display a high degree of off-target activity due to most viruses' innately broad tissue specificity (18). Many of these drawbacks can be mitigated through careful selection of delivery vehicle and controlled dosing. There has been heavy focus placed on developing more effective means of drug delivery and better delivery systems. Coupled with these expected advances in target-specific delivery, GDEPT and ADEPT have the promise to overcome shortcomings in classical prodrug therapy.

A variety of enzyme-prodrug combinations have been tested for GDEPT and ADEPT. The two most prominent systems are the cytosine deaminase (CD)/5-fluorocytosine (5-FC) and the Herpes simplex virus thymidine kinase (HSV-TK)/ganciclovir (GCV) combinations. 5-FC is a relatively non-toxic prodrug which is converted to the well known chemotherapeutic 5-fluorouracil (5-FU) by cytosine deaminase. Cellular metabolites of 5-FU can be incorporated into RNA and DNA to cause significant cytotoxicity (20). Humans do not possess a cytosine deaminase and cannot deaminate 5-FC readily. Delivering CD to a specific cell population generates target-specific toxicity once 5-FC has been administered. The system also possesses a large "bystander effect", where adjacent cells that lack CD experience cytotoxicity from 5-FU molecules that have been generated in cells harboring CD. The bystander effect can be advantageous or disadvantageous to a treatment, depending on the nature of the condition being treated. In tumors where targeted gene delivery is difficult and inefficient, a large bystander effect may balance out the small fraction of tumor cells that can be host to the gene delivery and still produce significant toxicity in the tumor area. However, a large bystander effect may also subject normal cells that are adjacent to cancer cells to high levels of cytotoxicity. The HSV-TK/GCV system is a well-studied enzyme-prodrug combination that produces a small bystander effect. GCV is a purine nucleoside analog that cannot be metabolized by human nucleoside kinases but can be activated by the promiscuous HSV-TK. Targeted delivery of HSV-TK prior to GCV administration results in specific cell populations experiencing cytotoxicity through DNA damage in a mechanism similar to 5-FU. However, to activate GCV, it needs to be phosphorylated instead of

deaminated; the charge on the metabolite makes it difficult for it to penetrate adjacent cells readily. This is the basis for GCV's low bystander effect. A measurable bystander effect is still observed due to gap junctions between cells that permit some transfer of charged metabolites (21).

HSV-TK & Nucleoside Analogs

The HSV-TK enzyme is a kinase that is encoded by the herpes simplex virus. The virus generally affects the mouth, face, central nervous system and eyes, although other sites of infection can also occur (22). Depending on the sites of infection, the virus can cause several medical conditions that can range from mild to severe, but the most common conditions are cold sores and skin lesions which can become painful. Severe HSV infections can be treated with antivirals that are designed to disable viral replication. Disrupting viral replication is a task that requires targeting the virus-specific machinery that is utilized during the replication process. HSV-TK is a key component of this process and is the first step in manufacturing thymidine triphosphate (dTTP). To date, almost every HSV-specific antiviral drug is a nucleoside analog that interacts with HSV-TK (23, 24).

The homodimeric, 376 amino acid long HSV-TK has a nucleoside binding site near its dimerization interface and a nucleotide binding site close to its exterior (Fig. 1.2). In contrast to most other thymidine kinases, which display high specificity for dT, HSV-TK is capable of phosphorylating dT, thymidylate (dTMP) and deoxycytidine (dC) (27, 57). The broad substrate specificity of the enzyme also allows it metabolize various nucleoside analogs along with these natural nucleoside

metabolites. This characteristic of HSV-TK enables the design of nucleoside analog prodrugs that can be exclusively phosphorylated by the enzyme.

Structural studies have shown that the high substrate tolerance comes from a large nucleoside binding site that can accommodate both pyrimidine and purine nucleoside analogs (25, 26). These structural studies have also revealed that while the large binding pocket allows many analogs to be to fit into it, the key interactions that take places for the catalysis are generally conserved (Fig. 1.3). Analysis of the active site shows that Q125 in the substrate-binding site forms two key hydrogen bonds to the substrate to help with orientation, regardless of whether it is a purine or pyrimidine (27). The residue E83 is responsible for accepting the proton from the 5' hydroxyl of thymidine and other nucleoside analogs during the creation of the phosphoester. Substrates are oriented through as base-stacking effect between Y172 and the purine or pyrimidine ring. Residue A163 is thought to be responsible for "locking in" the substrate for phosphorylation. Other key residues such as A168, A167 and D162 have been identified as playing roles in substrate binding and catalytic activity as well through mutational studies (23, 28, 29). Thermodynamic studies have revealed that the formation of a substrate-enzyme complex is necessary for adenosine triphosphate (ATP) to bind as the phosphate donor and catalyze the reaction (33). Interestingly, the enzyme lacks high-level homology towards other thymidine kinases but shares structural similarities towards other kinases such as porcine adenylate kinase and human cytidine kinase.

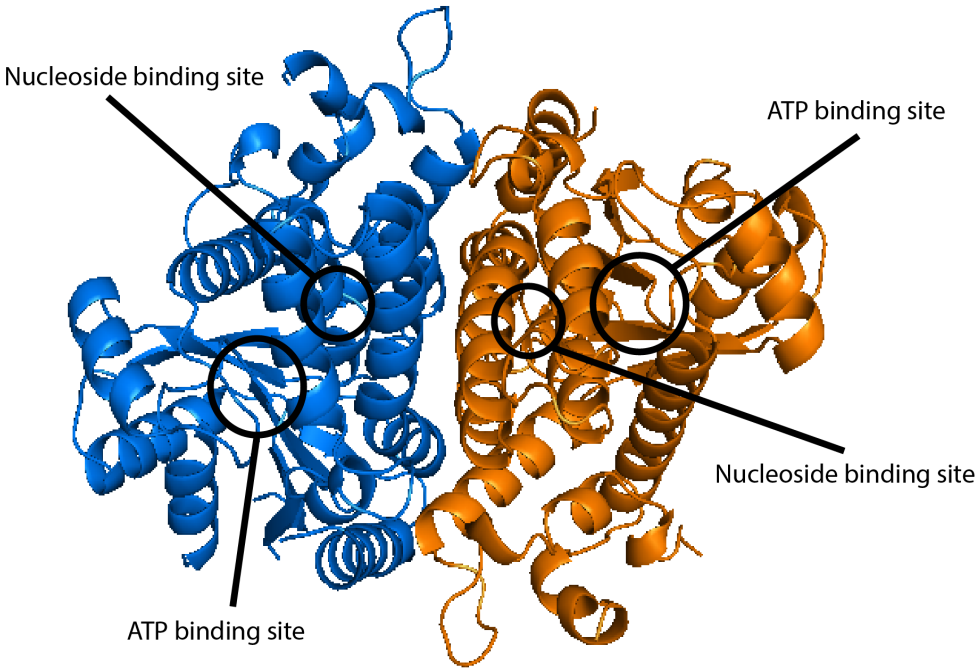

Figure 1.2: HSV-TK Enzyme

The homodimeric HSV-TK enzyme with each monomer colored differently (PDB #1KIM[58]). The ATP-binding sites and nucleoside binding sites are indicated for each monomeric unit. The nucleoside binding sites exists in between a triple helical fold while the ATP binding site consists with free loops that also interact with Mg^{+2} ions.

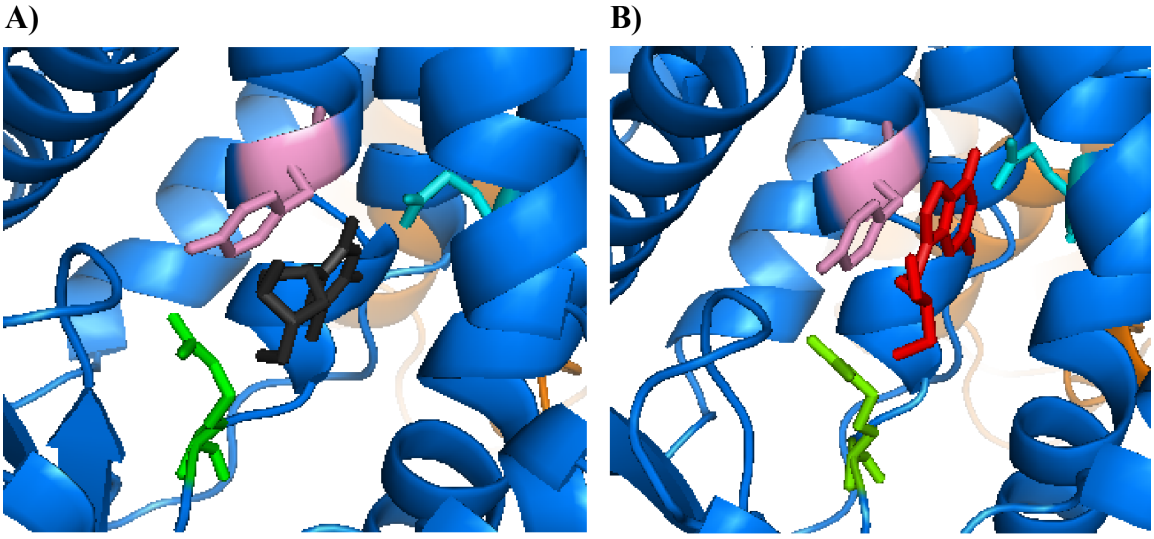

Figure 1.3: HSV-TK active site with dT & GCV

A) HSV-TK bound to dT (PDB #1KIM[58]). Orientation of dT (black) in the active site of HSV-TK with key interacting residues highlighted. Prominent base stacking is seen between Y172 (pink) and dT. The O5'of dT hydrogen bonds with R163 (green). This orientation allows two hydrogen bonds to be made between Q125 (cyan) and the N3 and O4 of the thymine ring. B) HSV-TK bound to GCV (PDB# 1KI2[58]). The orientation of the GCV (red) in the active site of HSV-TK is shown. The same interacting residues are highlighted. Base stacking between Y172 and GCV exists, although it is not as prominent as with dT. The O4 of GCV still forms a hydrogen bond with R163. Q125 still forms two hydrogen bonds with the purine ring's N1 and O6.

Table 1.1: List of Nucleoside Analogs that interact with HSV-TK

Nucleoside Analog	Analog Type	Class	Current Applications	IUPAC Name
Acyclovir	Purine	Antiviral	HSV-1, HSV-2, Chickenpox, Shingles, CMV	2-Amino-1,9-dihydro-9-((2-hydroxyethoxy)methyl)-6*H*-purin-6-one
Ganciclovir	Purine	Antiviral	HSV-1, HSV-2 CMV	2-amino-9-{[(1,3-dihydroxypropan-2-yl)oxy]methyl}-6,9-dihydro-3*H*-purin-6-one
Penciclovir	Purine	Antiviral	HSV-1, HSV-2, Shingles	2-amino-9-[4-hydroxy-3-(hydroxymethyl)butyl]-1*H*-purin-6(9*H*)-one
Azidothymidine	Pyrimidine	Antiviral	HIV	1-[(2*R*,4*S*,5*S*)-4-Azido-5-(hydroxymethyl)oxolan-2-yl]-5-methylpyrimidine-2,4-dione
Famciclovir	Purine	Antiviral	HSV-1, HSV-2, Shingles	2-[(acetyloxy)methyl]-4-(2-amino-9*H*-purin-9-yl)butyl acetate
Idoxuridine	Pyrimidine	Antiviral	HSV-1	1-[(2*R*,4*S*,5*R*)-4-hydroxy-5-(hydroxymethyl)oxolan-2-yl]-5-iodo-1,2,3,4-tetrahydropyrimidine-2,4-dione
Trifluridine	Pyrimidine	Antiviral	HSV-1	1-[4-hydroxy-5-(hydroxymethyl)oxolan-2-yl]-5-(trifluoromethyl) pyrimidine-2,4-dione
18F-FEAU	Pyrimidine	Imaging	PET Imaging	2'-deoxy-2'-[(18)F]fluoro-5-ethyl-1-β-D-arabinofuranosyluracil
18F-FIAC	Pyrimidine	Imaging	PET Imaging	1-(2'-deoxy-2'-[(18)F]fluoro-β-D-arabinofuranosyl)-5-iodocytosine
18F-FIAU	Pyrimidine	Imaging	PET Imaging	2'-deoxy-2'-[(18)F]fluoro-5-iodo-1-β-D-arabinofuranosyluracil
5-Fluorodeoxyuridine	Pyrimidine	Antimetabolite	Colorectal, Stomach, Skin, Breast, Pancreatic cancer	5-Fluoro-1-[4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl]-1H-pyrimidine-2,4-dione
Gemcitabine	Pyrimidine	Antimetabolite	Bladder, Ovarian, Lung cancer	4-amino-1-(2-deoxy-2,2-difluoro-β-D-*erythro*-pentofuranosyl)pyrimidin-2(1*H*)-on

The array of nucleosides and nucleoside analogs that can be phosphorylated by HSV-TK make the enzyme suitable to be used for a variety of functions. These nucleoside analogs have been implicated as antivirals, anticancer drugs and potential imaging agents (Table 1.1). Many of analogs were initially designed for application as antivirals for infections not related to HSV. For example, azidothymidine (AZT) was created and utilized as a frontline drug against HIV infection and ganciclovir was used for cytomegalovirus (CMV) infections. These drugs target disruptions in DNA and RNA synthesis to induce genotoxicity (30). The utility provided by HSV-TK also encouraged the development of radiolabeled nucleoside analogs such as ^{18}F-FEAU and ^{18}F-FIAC, which could potentially act as imaging agents to track infections and cancerous tumors in the body (59).

Protein Engineering of HSV-TK & Implications

The diverse applications of HSV-TK have driven efforts to tailor the enzyme towards specific functionalities. Protein engineering studies have resulted in HSV-TK variants with lower activity towards dT, higher activity towards prodrugs, and better understanding of key catalytic residues (28, 29). Many of these investigations were conducted in the early 1990s, when advanced mutagenesis techniques were still being developed and understanding of HSV-TK structural characteristics was still in its infancy. X-ray crystallographic structures of HSV-TK were first assembled in 1995 but employing them for rational protein engineering purposes was not possible due to the enzyme's limited homology to other studied proteins and inadequate understanding of protein perturbation effects. Therefore, directed evolution techniques were predominantly utilized in engineering HSV-TK. Methods

such as error-prone PCR and DNA fragment replacement were considered "best-in-practice" for creating mutagenic DNA libraries of HSV-TK variants (31). These libraries were subjected to screening and selections to identify variants that had higher activity towards nucleosides and nucleoside analogs. Through such processes, variants of HSV-TK were found that exhibited 4-fold increase in activity towards drugs like acyclovir (ACV) and a 40% reduction in activity towards dT (32). These studies also highlight potential epistatic affects of certain mutations in influencing kinase activity, such as the I160F mutation, which helped confer increased activity towards the nucleoside analogs GCV and ACV (34).

The success in engineering HSV-TK was particularly extraordinary due to limitations beyond the lack of thorough enzyme structure knowledge for rational design and availability of advanced mutagenesis techniques to efficiently create large libraries, such as comprehensive codon mutagenesis libraries. Selections of higher activity HSV-TK variants were primarily conducted by inhibiting endogenous dTMP production within *E. coli.* The two ways of producing dTMP in the bacteria are through the endogenous enzymes thymidine kinase and thymidylate synthase. Endogenous dTMP production can be prevented by genetically deleting thymidine kinase and inhibiting thymidylate synthase by the addition of 5-fluorouridine (5-FdU) to the growth media (32, 60). However, 5-FdU is a toxic compound that is a downstream metabolite of 5-FU. The compound is also a substrate of HSV-TK that only inhibits thymidylate synthase after being converted to its monophoshphate form, 5-FdUMP. The selections that were employed during these mutagenesis experiments effectively placed a "cap" on activity levels that could be selected for;

any variants that displayed extremely high levels of kinase activity would inadvertently generate high toxicity through 5-FdU phosphorylation.

There has been tremendous progress made in the last the ten years that should facilitate further engineering of HSV-TK in the future. Mutagenesis techniques have become more robust and efficient. Newer protocols allow for comprehensive, customizable mutagenesis that can be calibrated towards certain mutational specifications (35, 36). This can allow for a thorough mutational analysis study of the HSV-TK that may reveal previously unknown residue interactions and effects. A new selection scheme, which will be described in this dissertation, is now available to help select for high levels of kinase activity amongst variants in libraries. Screening has become dramatically faster due to the arrival of robotic screening, which has already been used to identify high activity HSV-TK variants (37). These advances in technology now allow HSV-TK to be engineered for specific functionalities more efficiently than ever before. The enzyme can be altered to have reduced activity towards dT while still being able to generate high toxicity in the presence of GCV. It can even be engineered to interact with more radiolabeled compounds like ^{18}F-FEAU to increase the diversity of agents that are available for position emission tomography (PET) scanning. Of particular interest to GDEPT and ADEPT technology, these advances enable to production of a "protein switch" of HSV-TK, which can have discriminating activity levels based on cellular environments.

Protein Switches

At its most basic level, a protein switch is a protein that can have its function regulated by effector molecule. Protein switches have evolved naturally and are involved in regulating key cellular functions such as signal cascades and transcription regulation. A classic example of a naturally occurring protein switch is the epidermal growth factor receptor (EGFR), which is found on surface of the cell. It primarily functions as a regulator of cell proliferation and is an important cancer marker. EGFR binds to a signal, epidermal growth factor (EGF), which causes a conformation change in the protein. This conformational change results in protein dimerization, which subsequently activates the EGFR's tyrosine kinase functionality that is located on the cytoplasmic side of the protein (38).

The mechanism of action of protein switches can differ but falls within two categories. The first is an allosteric model, wherein effector molecule binding drives a conformational shift in the protein to create alter the protein's level of activity. Most natural protein switches employ this model. The second category is a restabilization effect model. In this model, a protein switch exists in an unstable form that causes cellular enzymes to degrade it. The binding of an effector molecule stabilizes the structure of the protein switch and allows it to accumulate, leading to increased net activity. Both models have an underlying theme of population differentiation in them. The allosteric model assumes that the protein switch exists in different conformational states with certain states being more prevalent in the unbound state and that population distribution changes upon binding of the effector molecule (39). The restabilization model assumes that the protein switch exists in

an unstable form and the binding of the effector restores varying levels of stability to each protein switch that leads to a net increase in accumulation of the construct inside cells and results in a net increase in protein activity (43).

Methods to develop protein switches can vary depending on the functionality that is desired. For example, studies have exploited disulfide bridges in a protein switch to subject it to electrochemical activation using rational design (40, 41). Synthetic transcriptional regulation in the MAPK pathway was achieved by adding complimentary leucine zippers to positive or negative modulators and a scaffold protein (63). In other studies, switches were designed by mutually exclusive folding in which fused protein domains exist in a conformational tug-of-war that can be modulated by ligand binding (61, 62). More recently, S.S. Gambhir's laboratory split the HSV-TK enzyme into two fragments that could dimerize and create an active protein (64). Their work showed the potential for using the split protein to generate localized, target specific signals in positron emission tomography imaging. Other studies created maltose and hypoxia-sensing enzymatic switches using a domain insertion strategy through directed evolution (42, 43). Domains that bind to the signal are inserted into the amino acid backbone of the enzyme in this strategy to sensitize the enzymatic activity of the protein to the signal. Binding of the effector to the domain results in an allosteric or stabilization effect that influences enzymatic activity.

In the past, domain insertion strategies in directed evolution involved using nucleases to create random breaks in the plasmid housing an enzyme's gene and then ligating the domain gene into those broken gene sequences (43). This protocol

is inefficient because the nuclease cannot specifically target sites in between codons to cleave and so, only 1 out of 3 digested genes would have the correct open reading frame after the domain has been ligated in. The issue is further compounded when taking into account that the genes are on plasmids, which means the nuclease can cleave sites outside of the gene. A more effective method of domain insertion that is capable of selectively opening up desired insertion points within a gene has been developed in the past few years (36). This method utilizes codon-targeting oligonucleotides in an inverse PCR reaction to linearize the plasmid of the gene by "opening up" the gene after a specific codon (Fig. 1.4). This can be done for every desired insertion point in the gene. A domain insertion strategy is particularly useful in engineering the therapeutically relevant enzymes utilized in GDEPT and ADEPT because it can sensitize the prodrug activating-enzymatic activity towards specific cellular states. This, in effect, can increase specificity of the targeted GDEPT/ADEPT treatment.

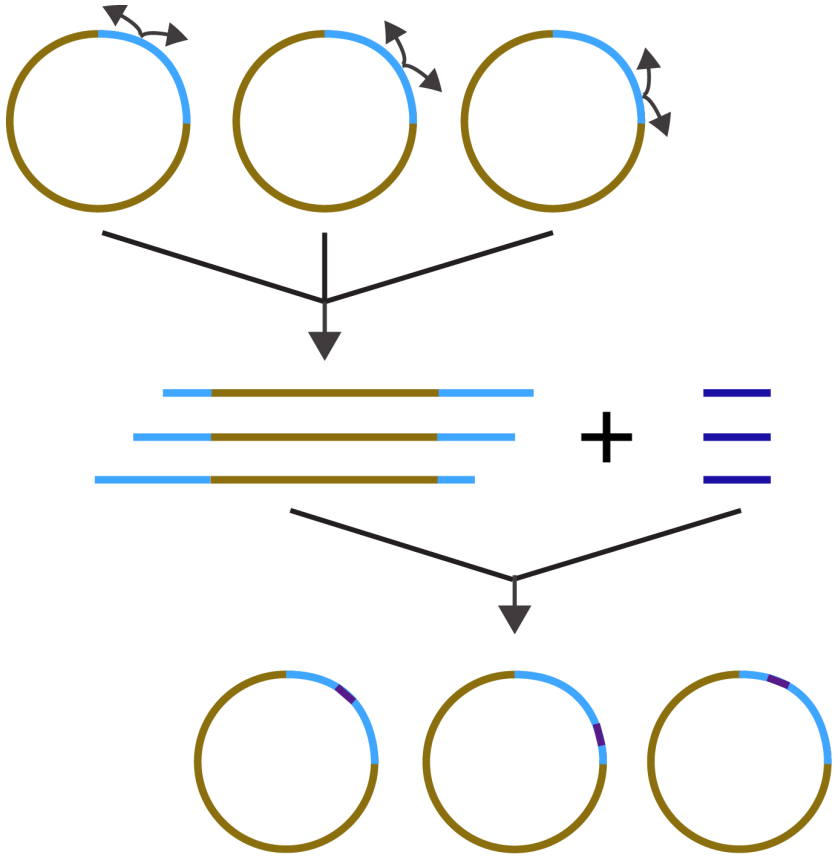

Figure 1.4: Domain insertion libraries by inverse PCR[36].

Domain insertion libraries are created by PCR amplifying linear plasmid molecules with "outward facing," non-phosphorylated primers that target specific codons that are desired as insertion points. The resulting linearized plasmids from the PCR step are ligated with the DNA encoding the insert protein. This re-circularizes the plasmids and inserts the insert gene in the targeted insertion points. This ensures all fused constructs are in the same open reading frame. Adding linkers to the 5' and 3' end of the insert gene before the ligation step can generate additional library diversity.

Selective prodrug-activating protein switches

Our lab has a major focus on developing these GDEPT and ADEPT compatible protein switches. We have previously developed enzymatic switches for yeast cytosine deaminase (yCD) that are capable of being activated by a cancer marker (43, 44). The design of the switches was based on three separate components. The first component is yCD, which functions as the signal output domain. The yCD enzyme is a 158 amino acid long homodimeric protein found in yeast, where it functions as a nucleotide salvage enzyme (45). The catalytic function of yCD is identical to the previously described activity of CD, however, a thermostabilized variant of yCD was utilized in these studies to promote the generation of stable switches (53). The second component is a CH1 domain, which acts as the signal recognition domain of the switch. Originally a part of the p300 protein, the CH1 domain recognizes a multitude of transcription factors and helps the protein conduct transcriptional regulation (47). The domain is capable of binding to HIF1-α, Stat2, CITED2, MDM2, p53, Ets-1 and NFκB (48-52). The final component of the switches is the signal molecule, HIF1-α. HIF1-α is a large hypoxia sensing protein that is comprised of several functional domains. These domains include the N-terminal transactivation domain (N-TAD), which is involved DNA and HIF1-β binding, along with a C-terminal transactivation domain (C-TAD) that recruits transcriptional co-regulatory proteins and binds to the CH1 domain of the p300 protein.

In normoxia, HIF1-α is constitutively degraded through the von Hippel-Lindau (VHL) protein. This degradation is inhibited in hypoxic conditions and HIF1-

α is able to dimerize with HIF1-β; this dimerization allows HIF1 to activate hypoxia related genes that promote processes such as angiogenesis and energy metabolism (54). The interaction between HIF1-α and CH1 is of particular interest since it implicates the CH1 domain in hypoxia response, a condition that is prevalent in some tumors. Nuclear magnetic resonance (NMR) studies have shown that the CH1 domain exists in a molten globule state in its isolated form but reverts to a stable, tri-helical structure when bound to HIF1-α (46). This structured state has also been seen in x-ray crystallographic structures of the CH1 domain binding to the C-TAD domains of HIF1-α and CITED2 (55, 56). The generated switches for yCD followed the stabilization model described earlier, possibly due to this structural state transition. It is interesting to note that while CITED2 is a direct inhibitor of HIF1-α binding with the CH1 domain and the CH1 domain adopts almost identical structures when bound to either C-TAD, the binding interfaces differ between the two (Fig. 1.5).

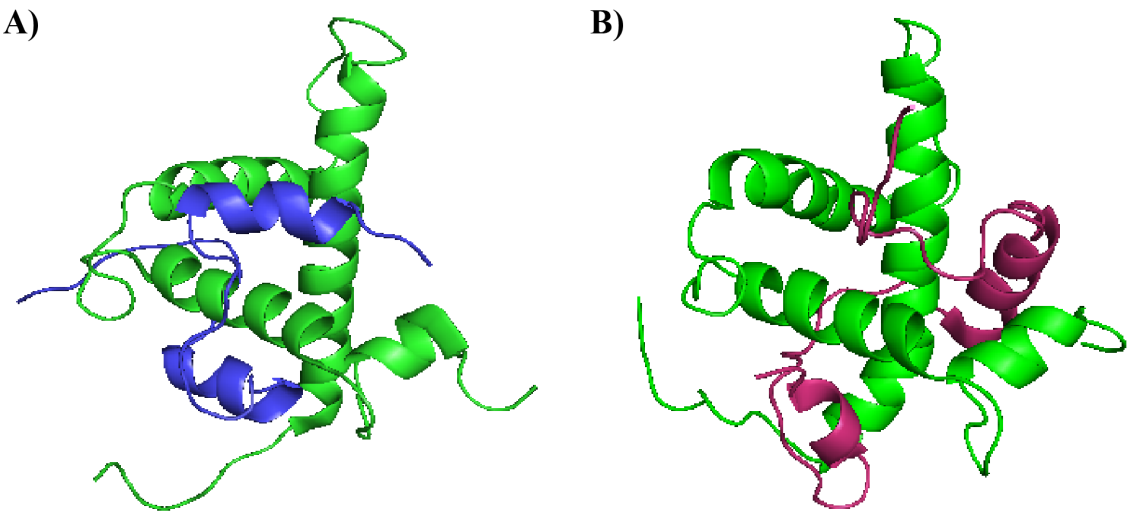

Figure 1.5: CH1 binding with C-TAD of HIF1-α & C-TAD of CITED2

A) The CH1 domain (green) bound to the C-TAD of HIF1-α (blue) (PDB# 1L3E[55]). The CH1 domain exists in a tri-helical conformation when bound the C-TAD. NMR studies have revealed that in the absence of binding, the domain adopts a partially unfolded structure. HIF1-α's C-TAD has two alpha helices sandwiching one specific alpha helix of the CH1 domain. B) The CH1 domain bound the C-TAD of CITED2 (purple) (PDB #1P4Q[56]). The binding interface is quite different from that of C-TAD and CH1; CITED2 interacts almost exclusively with the smallest alpha helix and largest alpha helices of the CH1 domain.

Engineering cancer-marker activated protein switches using the CH1 domain as a signal recognition domain and the C-TAD of HIF1-α as the signal has shown to be achievable. Despite this, the multitude of binding partners that CH1 is potentially a problem in other CH1 binders in some normal cells may activate the switch. Most of these binding partners, however, are proteins found to be upregulated in cancer cells and cumulative effect of non-target binding may beneficial.

Many GDEPT studies have shown the synergistic effects of using HSV-TK/GCV and yCD/5-FC treatments simultaneously. Our lab has previously focused

on engineering yCD to help make GDEPT treatments more cancer specific. This thesis highlights progress made towards advancing HSV-TK's applicability for cancer treatment, including the development of a cancer activated HSV-TK protein switch and a new method of engineering kinases more effectively.

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Chapter 2: A positive selection for nucleoside kinases in *E. coli*

Summary:

Engineering heterologous nucleoside kinases inside *E. coli* is a difficult process due to the integral role nucleosides play in cell division and transcription. Nucleoside analogs are used in many kinase screens that depend on cellular metabolization of the analogs. However, metabolic activation of these analogs can be toxic through disruptions of DNA replication and transcription because of the analogs' structural similarities to native nucleosides. Furthermore, the activity of engineered kinases can be masked by endogenous kinases in the cytoplasm, which leads to more difficulties in assessing target activity. A positive selection method that can discern a heterologous kinases' enzymatic activity without significantly influencing the cell's normal metabolic systems would be beneficial. We have developed a means to select for a nucleoside kinase's activity by transporting the kinase to the periplasmic space of an *E. coli* strain that has its PhoA alkaline phosphatase knocked out. Our proof-of-principle studies demonstrate that the herpes simplex virus thymidine kinase (HSV-TK) can be transported to the periplasmic space in functional form by attaching a *tat*-signal sequence to the N-terminus of the protein. HSV-TK phosphorylates the toxic nucleoside analog 3'-azido-3'-deoxythymidine (AZT), and this charged, monophosphate form of AZT cannot cross the inner membrane. The translocation of HSV-TK provides significant resistance to AZT when compared to bacteria lacking a periplasmic HSV-TK.

However, resistance decreased dramatically above 40 μg/ml AZT. We propose that this threshold can be used to select for higher activity variants of HSV-TK and other nucleoside kinases in a manner that overcomes the efficiency and localization issues of previous selection schemes. Furthermore, our selection strategy should be a general strategy to select or evaluate nucleoside kinases that phosphorylate nucleosides such as prodrugs that would otherwise be toxic to *E. coli*.

Introduction:

Nucleoside kinases are key enzymes in bacterial and mammalian cell reproduction. Because of their involvement in metabolizing nucleosides and maintaining nucleotide pools, nucleoside kinases are major regulators of cellular processes. Nucleoside and nucleotide levels are extensively involved in events such as cell replication, DNA and RNA synthesis, cell signaling and stress response. Since they play such significant roles in essential processes, the last few decades have seen a surge in the development of nucleoside analogs as anticancer and antiviral agents (3). These nucleoside analogs work by hijacking the machinery of the host cell or virus to inhibit the proper function of processes like DNA or RNA synthesis, eventually leading to the destruction of the host (3). Anticancer and antiviral nucleoside analogs differ in that antiviral analogs are designed to have lower toxicity towards mammalian systems (3). Most viruses have their own nucleoside kinases, and the chemical design of antiviral agents is targeted to make these agents substrates of those specific kinases (3, 4). Since most viral nucleoside kinases share little homology towards mammalian or bacterial kinases, the antiviral analogs are not as easily metabolized by eukaryotic or prokaryotic systems. This key difference

provides a higher dose tolerance for antiviral agents but also makes their usefulness restricted to viral infections.

However, advances in targeted gene delivery have shown some promise and may provide a way to implement antiviral agents as anticancer agents. If mammalian cells, such as cancer cells, are targeted to express these non-mammalian genes, the cells obtain the ability to activate antiviral prodrugs (5). This causes toxicity in the targeted mammalian cells as the metabolized antiviral agents cause obstructions in DNA and RNA synthesis (5). The development of better gene delivery systems has led to a resurgence in the engineering of non-mammalian proteins for gene therapy and medical imaging purposes. Focus has increased in engineering nucleoside kinases, such as the herpes simplex virus thymidine kinase (HSV-TK) (EC 2.7.1.21), with higher activity towards antiviral prodrugs and medical imaging analogs such as 3'-azido-3'-deoxythymidine (AZT) and [^{18}F]-2′-fluoro-2′deoxy-1h-D-arabionofuranosyl-5-ethyl-uracil (^{18}F-FEAU) (6-9). However, the paucity of methods for selecting for engineered nucleoside kinases in a high-throughput manner presents a limitation for their engineering.

There are two key factors that make engineering nucleoside kinases difficult. First, selecting for nucleoside kinase activity traditionally involves utilizing a toxic nucleoside analog (6, 8, 10). For in vivo selections, phosphorylation of the analog by the engineered kinase results in the toxic effects of the analog's metabolites, which obstruct DNA and RNA synthesis (11, 12). When the concentration of these metabolized nucleoside analogs are high enough, the resulting obstructions eventually lead to cell death and thereby place strict limitations on the activity

windows that can be found for engineered kinases in the selection (13). Second, it is difficult to separate engineered nucleoside kinase activity from endogenous nucleoside kinase activity for in vivo selections. This adds to the complexity in identifying higher activity variants in direct evolution selections and requires additional screens to be able to clarify the efficacy of an engineered variant (14).

Here, we have developed a bacterial positive selection for nucleoside kinases that addresses both of these issues. The selection involves exporting the HSV-TK to the periplasm of *E. coli* BW14012 strain (*phoA*$^-$). The HSV-TK protein has a tat-signal sequence attached to its N-terminus. After translation and folding of the kinase in the cytoplasm, the *tat*-signaling pathway recognizes the signal sequences and exports the fully-folded protein to the periplasmic space of the bacteria (2). Upon translocation, periplasmic peptidases cleave the signal sequence off (2). In the periplasm, HSV-TK retains its kinase activity and is capable of phosphorylating extracellular nucleosides and nucleoside analogs (15). In the presence of AZT, the kinase phosphorylates the nucleoside analog, thereby preventing the molecule from crossing the inner membrane of the cell. Inside the cell, AZT and AZP-MP would be further metabolized and inhibit DNA and RNA synthesis. Thus, periplasmic kinase activity prevents toxicity and allows the cell to survive in this positive selection.

There are two specific benefits to sending a heterologous kinase to the periplasm of *E. coli* for protein engineering. First, by exporting the protein to the periplasm, its activity is spatially segregated from endogenous kinases in the cytoplasm. This helps reduce ambiguity about the source of kinase activity being from the engineered protein or endogenous enzymes. Second, nucleosides and

nucleoside analogs that are phosphorylated by the kinase in the periplasmic space cannot breach the inner membrane. In normal cells, the intake of nucleoside analogs, such as the prodrug AZT, is followed by its cytoplasmic phosphorylation via nucleoside kinases (16). This phosphorylation event instigates the metabolism of the nucleoside analog in to its triphosphate form and eventual incorporation into DNA or RNA, through which it provides its toxicity (14). The addition of a charged moiety in the periplasm inhibits the nucleoside analog from crossing the inner membrane and thus, the bacterium becomes resistant to the drug. The deletion of the PhoA in *E. coli* BW14012 ensures that phosphorylated nucleosides in the periplasm are not dephosphorylated back in to their original, transport competent form (17, 18).

Materials & Methods:

Kits & Reagents:

Unless otherwise specified, all molecular biology protocols were performed using NEB's High-Fidelity Phusion Master Mix for PCR, Invitrogen's Gel Purification Kit for gel extraction and Zymo's DNA Clean & Concentrator kit for DNA purification (5 μg loading capacity). Qiagen's QIAprep Spin Miniprep Kit was used to isolate plasmid DNA from cell culture. 3'-Azido-3'-Deoxythymidine (Azidothymidine, AZT) was purchased from Sigma-Aldrich. 5'-Triphosphate -3'-Azido-3'-Deoxythymidine (AZT-TP) was obtained through US Biological Life Sciences. All nucleotide oligomers and gBlocks were ordered from Integrated DNA Technologies. Ligations were

performed using New England Biolabs' T4 DNA Ligase Buffer (10x) and T4 DNA Ligase (400,000 units/mL).

Strains:

E. coli strains BW14012 (*F^- , Δ(codB-lacI)3, ΔphoA532, Δ(phnJ-mel)524(Tn5-1/132), [phn_B])* and W3110 *(F,$^-$ lambda$^-$, IN(rrnD-rrnE)1, rph-1)* were obtained from the Coli Genetic Stock Center at Yale University. *E. coli* 5α *(fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17)* was purchased from New England Biolabs.

pSkunk2-hsvtk Plasmid Construction & active site deletion:

The original *hsv-tk* gene was provided by Dr. Margaret Black (University of Washington) in a plasmid dubbed "pMCC". All experiments were conducted using the pSkunk2 plasmid, which was previously developed in the lab (19). Primer pairs binding to the beginning of *hsv-tk* and the end of the *hsv-tk* gene in pMCC were designed. The forward primer that overlapped the start codon of *hsvtk* had a flanking NcoI restriction site (5-ATATTAACCATGGATGGCTTCGTACCCCTGCCATC-3') and the reverse primer that overlapped the stop codon of *hsvtk* had a SalI restriction site (5'-ATATTAACCATGGATGGCTTCGTACCCCTGCCATC-3'). PCR was performed using the pMCC vector as a template with the forward and backward primers. The protocol utilized an initial 2 min 98°C denaturation step, followed by 25 cycles of 98°C denaturation for 30 seconds, 57°C annealing step for 20 seconds, 72°C elongation step for 1 min. There was a final 5 min. 72°C elongation step to finish the amplification. The amplified *hsv-tk* was then gel extracted and concentrated.

pSkunk2 was purified from *E. coli* 5α by using a miniprep kit to get isolated plasmid. The low copy plasmid contains a *tac* promoter inducible with isopropyl β-D-1-thiogalactopyranoside (IPTG) and provides streptomycin resistance through an *aadA* gene. Both the isolated plasmid and the amplified *hsv-tk* were then individually subjected to a double restriction digest step. In a 20 μL reaction, 1 μg of plasmid or *hsvtk* was added to NEB's CutSmart buffer (10x), SalI-HF (NEB), NcoI-HF (NEB) and deionized water. The digestion was run for 1 hour, after which the DNA was purified.

The resulting products of linearized, digested pSkunk2 and *hsvtk* were then ligated together to finish cloning the gene in to the plasmid. The DNA ligation used 100 ng of linearized plasmid with 100 ng of the linearized, digest *hsvtk* gene (approximately 1:3 plasmid to insert ratio) in a 20 μL reaction. The appropriate amounts of T4 DNA Ligase & Buffer were added as recommended by NEB. The reaction was conducted for 1 hour and then purified for DNA. Two μL of the purified DNA ligation was transformed in to *E. coli* 5α using electroporation. Colonies from the transformation were sent for sequencing to confirm proper insertion.

Confirmed pSkunk2-*hsvtk* was then subjected to a deletion step using restriction enzymes to yield an inactive *hsvtk* variant. The pMCC plasmid underwent KpnI and SacI digestion, which removed 77 base pairs from the *hsv-tk* gene. A ligation with a small nucleotide sequence with corresponding KpnI and SacI flanks (Forward: 5'-CCCCTCGAGCGCGGTAC-3', Reverse: 5'-CGCGCTCGAGGGGAGCT-3') recircularized the plasmid and resulted in an *hsv-tk* with a 66 base pair deletion in

its active site. This inactivated form of *hsv-tk* has been used before as a negative control (20). The inactive *hsv-tk* gene was dubbed "*hsv-tkΔ*".

Addition of signal sequences HSV-TK:

Circular polymerase extension cloning (CPEC) was used to add three signal sequences to the front of the start codon of *hsvtk* on pSkunk2. Oligonucleotides targeted to linearize pSkunk2-*hsvtk* from the start codon of the gene were ordered (Forward: 5'-ATGGCTTCGTACCCCTGCCA-3', Reverse: 5'-CCATGGATCCTTCCTCCTGTGT-3'). PCR was performed to linearize the plasmid, with an annealing step of 57°C annealing step for 20 seconds and 72°C elongation step for 1:50 min. The product was gel extracted and purified. Oligonucleotides containing a signal sequence and flanks homogenous to each end of the linearized plasmid were ordered for all three signal sequences (*supplementary*). CPEC was conducted using the linearized DNA fragments according to a previously established protocol (21). Five μL of CPEC reactions were transformed into chemically competent *E. coli* 5α cells (New England Biolabs) and the resulting colonies sequenced for insertion confirmation.

AZT toxicity assays:

pSkunk2-*hsvtk* constructs with the three signal sequences were transformed into *E. coli* BW14012. Approximately 2500 CFU (determined in the absence of AZT) of cells harboring each construct were plated on LB Agar plates containing 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG), 2.5 mM adenosine triphosphate (ATP), 50 μg/mL streptomycin and 0, 5, 10, 20, 30, 40, 50 or 60 μg/mL AZT. The

plates were incubated for 20 hours at 37°C and the percent viability was calculated as the number colonies on each plate divided by the number of colonies on the plate with 0 μg/mL AZT. Assays with *E. coli* W3110 and 5α strains were performed in the same manner.

Western blot of periplasmic and cytoplasmic fractions:

E. coli BW14012 were grown overnight expressing *dsbA-hsvtk* (dsbHSV-TK), *pelB-hsvtk* (pelHSV-TK), *tat-hsvtk* (tatHSV-TK) or inactive *tat-hsvtk* (tatHSV-TKΔ). The optical densities (OD) normalized and the cells subjected to osmotic shock to collect the periplasmic fraction (22). Cultures were pelleted through centrifugation and then resuspended in cold sucrose buffer (50 mM Tris-HCl (pH 7.4) with 1 mM EDTA and 20% sucrose). Half the culture volume was used for resuspension. The cells were then shaken on ice for 10 minutes and then pelleted again through centrifugation at 4°C. The supernatant was removed and the pelleted cells were resuspended in cold 5 mM $MgCl_2$ solution (in a fourth of the culture volume). The resuspended cells were shaken on ice for 15 minutes and centrifuged down again at 4°C. The supernatant contained the periplasmic fraction and was collected. The pelleted cells were then subjected to cell lysis using Novagen's BugBuster agent to collect the cytoplasmic fraction. The protocol for lysis was conducted as indicated in the kit and soluble fractions were collected.

Periplasmic and cytoplasmic fractions of each variant were electrophoresed on a NuPage 4-12% Bis-Tris protein gel (ThermoFisher Scientific) using SDS-PAGE. The proteins were then transferred on to a polyvinylidene fluoride (PVDF) membrane using Biorad's Trans-Blot SD Semi-dry Transfer Cell for 15 minutes at

15V. The primary antibody against HSV-TK (Santa Cruz Biotechnologies, Inc.) was incubated with the membrane after protein transfer in Tris-buffered saline-Tween (TBST) buffer at a 3:500 antibody:buffer ratio for 1 hr at 4°C. The anti-goat secondary antibody (Biorad Laboratories, Inc) was loaded on to the western blot in TBST buffer at the same ratio as the primary antibody. No wash steps or blocking steps were performed. We visualized the western by adding HRP substrate using Biorad's Clarity Western ECL Substrate as per the protocol provided. The chemiluminescence was observed using Biorad's Universal Hood II and Quantity One Software.

Selection enrichment assay:

BW14012 cells harboring wild-type *hsv-tk (tat)* were mixed with cells holding *hsvtkΔ (tat)* in a 1:10000 ratio. A total of 500,000 CFU of the mixture was plated on large bioassay plates containing LB Agar, IPTG (1 mM), ATP (2.5 mM), streptomycin (50 μg/mL) and AZT (40 μg/mL). The plates were incubated for 24 hours at 37°C. Colonies were subjected to a colony screening PCR to determine which form of *hsvtk* they harbored. There was a 66 bp size difference between wild-type *hsv-tk* and the smaller, inactive *hsv-tk.* The PCR protocol was identical to the one described earlier for *hsvtk* amplification and the same primers were used. After analysis of DNA size by agarose gel electrophoresis, the plasmids of five colonies were sequenced. The minimum enrichment rate was found by dividing the ratio of HSV-TK positive colonies to total colonies by the mix ratio (1:10,000).

Results:

We used HSV-TK as the kinase and AZT as the prodrug (Fig. 2.1) in our proof-of principle studies. We first sought to test the underlying assumptions that phosphorylated AZT cannot breach the inner membrane and that phosphorylated AZT monophosphate (AZT-MP) is much less toxic than AZT when supplied in the growth media. Since AZT-MP is not available commercially, we substituted AZT triphosphate (AZT-TP) to act as a proxy. Bacterial outer membranes allow small molecules to pass through using either a lipid-mediated pathway or through passive diffusion of hydrophilic molecules up to 600 Daltons using porins (1,2). We presumed that AZT (267 Daltons) and AZT-TP (507 Daltons) utilize the latter. We challenged *E. coli* DH5α cells to grow on solid media containing AZT or AZT-TP. Whereas AZT prevents growth at just 0.5 μg/ml, AZT-TP could be present up to at least 10 μg/ml media without any decrease in cell viability. This is consistent with the notion that the phosphorylated molecule is not toxic to cells because it cannot penetrate the inner membrane.

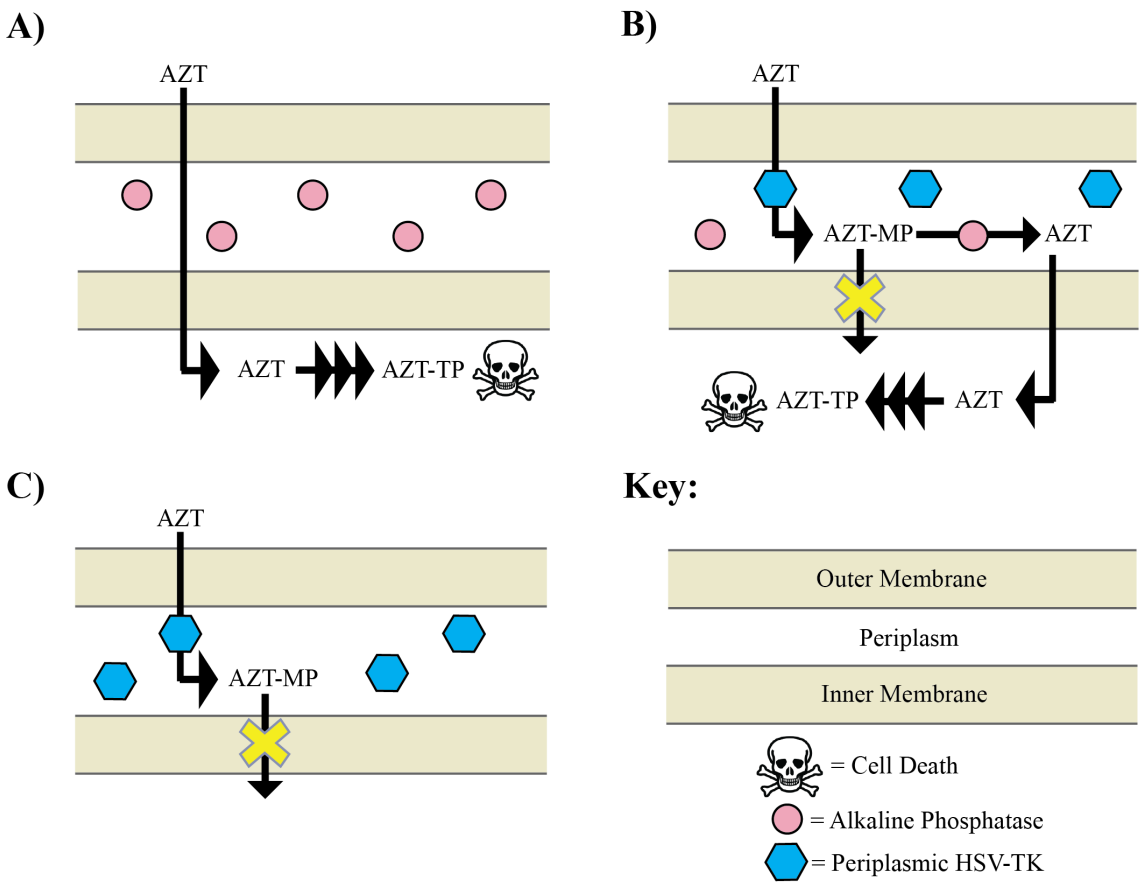

Figure 2.1: Positive selection for HSV-TK activation of AZT. A) In typical cells, AZT penetrates the outer membrane, periplasm and inner membrane to be metabolized in to its triphosphate form to generate genotoxicity towards *E. coli.* B) When HSV-TK is exported to the periplasm AZT is phosphorylated in the periplasm; however, PhoA counteracts by dephosphorylating the AZT-MP, allowing AZT to penetrate the inner membrane causing genotoxicity. C) In cells lacking PhoA but containing periplasmic HSV-TK, AZT is phosphorylated and AZT-MP cannot cross the inner membrane. Thus, periplasmic HSV-TK provides AZT resistance to *phoA*$^-$ strains.

Periplasmic transport of HSV-TK:

For the selection to work properly, HSV-TK must be exported to the periplasm in functional form. Previous studies have confirmed the presence of HSV-TK in the periplasm through western blot analysis of isolated, soluble periplasmic

fractions (13). The study utilized a *pelB* signal sequence to export HSV-TK to the periplasm in *Salmonella typhimurium* but did not test other export signal sequences. We expected that efficient transport of HSV-TK into the periplasm would generate stronger resistance towards AZT. To identify the most efficient methods of transport *E. coli*, we tested three separate periplasmic-export signal sequences. The *pelB* and *dsbA* signal sequences export unfolded protein into the periplasmic space using the Sec pathway and SRP pathway, respectively (23). The *tat* signal sequence sends folded proteins into the periplasm using the twin-arginine pathway (24). We fused the wild-type *hsv-tk* gene to the *dsbA*, *pelB,* and *tat* export signal sequences on the pSkunk2 plasmid and transformed into the *phoA*⁻ *E. coli* strain BW14012 (19). Cells harboring each construct were plated on solid media containing 2.5 mM ATP and different concentrations of AZT. We added ATP because HSV-TK transfers the phosphate from ATP, and ATP is not naturally present in the periplasm. The *tat* signal sequence provided robust resistance even at 10 μg/ml AZT (Fig. 2.2a). The *pelB* and *dsbA* signal sequences provided no resistance, as no growth was observed even at 1 μg/ml AZT. In contrast, high levels of AZT resistance (up to 40 μg/mL) were observed for cells containing wild-type HSV-TK with a *tat*-signal sequence (tatHSV-TK). Export of a mutated, inactive version of HSV-TK (tatHSV-TKΔ) via the Tat pathway provided no AZT resistance. This inactive, mutated version of HSV-TK is previously described (20) and contains a deletion in the active site of the enzyme.

To confirm that HSV-TK was being successfully transported into the periplasm, we performed a western blot on the periplasmic and cytoplasmic fractions of *E. coli* BW14012 expressing tatHSV-TK, dsbHSV-TK or pelHSV-TK (Fig.

2.2b). An catalytically inactive HSV-TK control with a tat signal sequence, tatHSV-TKΔ, was also tested for proper transport. Both HSV-TKs with the tat signal were present in the cytoplasmic and periplasmic fractions but HSV-TK conjugated to the pelB or dsbA signal sequences were not detected in either fraction. These results indicate the necessity for folded protein translocation for HSV-TK in order to provide AZT resistance.

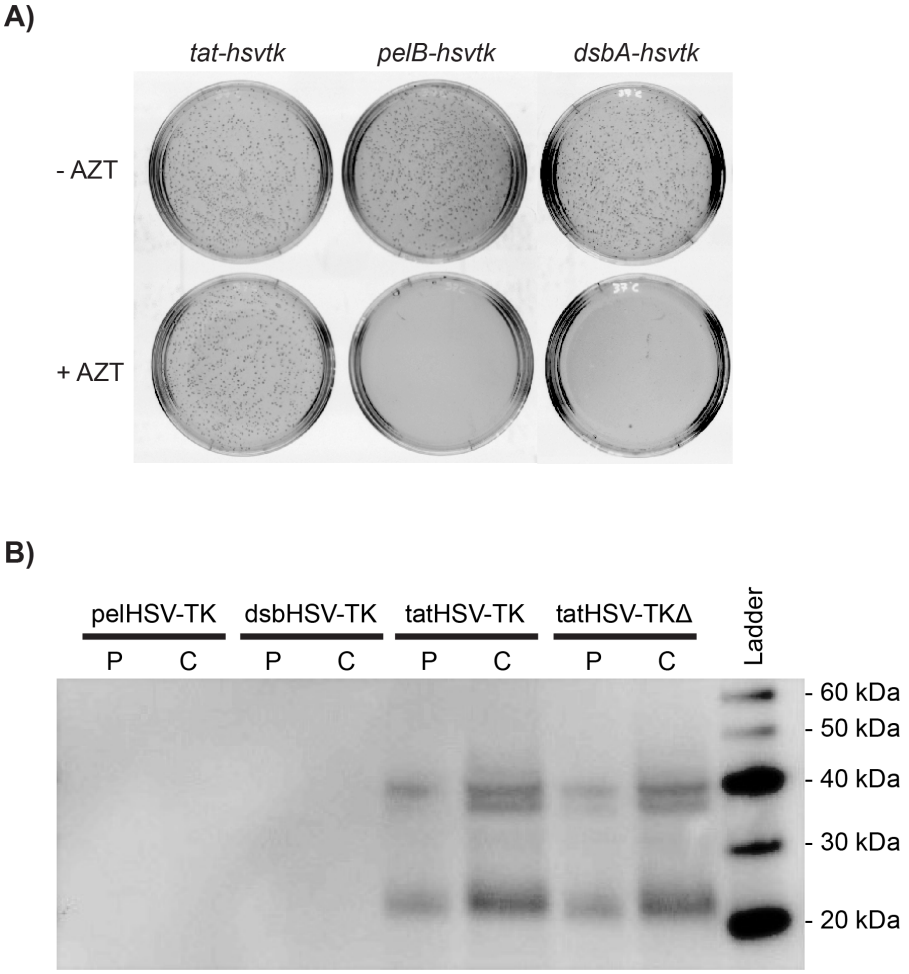

Figure 2.2: Export of active HSV-TK using the tat signal sequence. A) 2500 CFUs of *E. coli* BW14012 cells with *tat-hsvtk, pelB-hsvtk or dsbA-hsvtk* were plated on LB agar plates containing 50 μg/mL streptomycin, 2.5 mM ATP, 1mM IPTG, and the presence (bottom row) or absence (top row) of 10 μg/mL AZT. The plates were incubated for 20 hours at 37°C. B) Western blot using anti-HSV-TK antibodies of periplasmic ("P") and cytoplasmic ("C") fractions of cells expressing the indicated proteins. The expect size of HSV-TK is 41 kDa.

Knock out of PhoA activity is essential for the positive selection:

In the preceding experiment, we used a strain of *E. coli* deficient in the periplasmic phosphatase PhoA. In theory, PhoA could remove phosphate groups from AZT that had been phosphorylated by HSV-TK. This would allow the molecules to pass through the inner membrane through nucleoside transporters and prevent the cell from possessing strong resistance to nucleoside analogs (25). We tested the importance of using a *phoA*$^-$ strain for the positive selection by comparing it to strains with a functional PhoA. We tested the AZT sensitivity of cells expressing wild-type and inactive HSV-TKΔ (both fused to Tat signal sequences) in the *phoA*$^+$ strains 5α and W3110. Strains 5α and W3110 showed much less resistance than the *phoA*$^-$ BW14012 strain. Expression of tatHSV-TK in 5α and W3110 provided only marginal resistance to AZT. Only 16% and 18%, respectively, of the cells plated were able to grow in the presence of 1 μg/mL AZT, and no colonies for either strain formed on plates containing 10 μg/ml AZT. In contrast, BW14012 cells expressing tatHSV-TK showed no growth defect at 10 μg/ml AZT and had 65% viability even at 40 μg/ml. We postulate that the substantial toxicity of AZT in 5α and W3110 is due to PhoA dephosphorylating AZT-MP, allowing it to enter the cell. Although 5α and W3110 are not isogenic to BW14012, we believe it is likely that the lack of PhoA in *E. coli* BW14012 is a crucial component of the positive selection.

AZT toxicity assays:

By comparing the magnitude of AZT resistance in the presence of periplasmic kinase and an inactivated counterpart, it was possible to determine the optimal conditions for a positive selection. We quantified cell viability on solid media as a function of AZT concentration for cells expressing tatHSV-TK and cells expressing the inactive tatHSV-TKΔ, both in *E. coli* BW14012. Cells with tatHSV-TK showed cell viability of 65 ± 2% at 40 μg/mL AZT while those with tatHSV-TKΔ had <0.01% viability even at 1 μg/mL AZT (Fig. 2.3). At concentrations up to 20 μg/mL AZT, almost full (>95%) viability was observed with cells expressing tatHSV-TK. After 20 μg/mL AZT, there was a decline in cell viability until 40 μg/mL AZT. The cells exhibited a drastic reduction in viability beyond this concentration of AZT. This drop represents a "resistance threshold" that wildtype HSV-TK kinase activity cannot overcome, but one that an improved HSV-TK might.

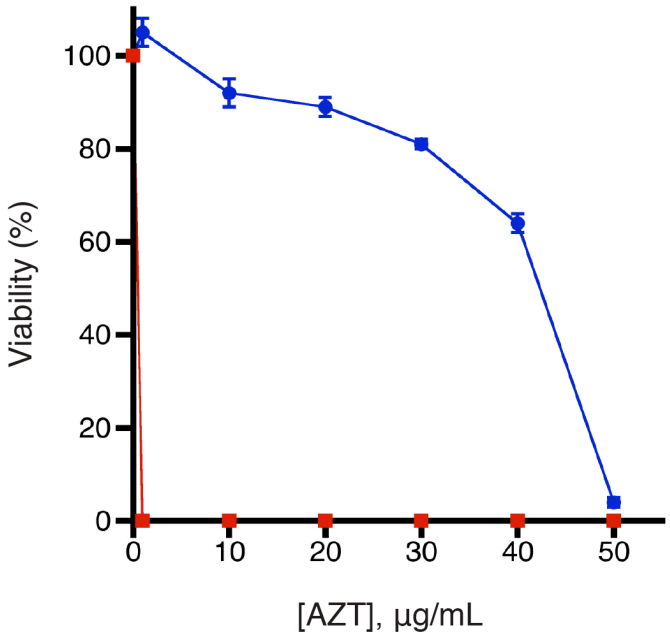

Figure 2.3: Viability of *E. coli* BW14012 cells expressing tatHSV-TK (blue circles) or tatHSV-TKΔ (red squares) as a function of AZT concentrations. Cell viability is expressed relative to the number of colonies at 0 µg/mL AZT for each strain type.

Demonstration of the selection:

We expect that the drastic drop in viability in *E. coli* BW14012 with periplasmic HSV-TK around 40 µg/mL AZT will allow for a positive selection that can identify engineered HSV-TK with increased kinase activity. We performed a mock selection experiment to test how effectively our positive selection can identify high activity kinases over kinases with less activity. The mock selection involved plating mixtures of BW14012 cells expressing tatHSV-TK or tatHSV-TKΔ (1:10,000 ratio) on solid media containing 40 µg/mL AZT. We plated 500,000 CFUs on solid media and 20 colonies formed. With a 1:10,000 mix ratio and a 65% observed viability for cells expressing tatHSV-TK at this AZT concentration, we expected approximately 33 colonies. Colony PCR was conducted to identify whether these

colonies harbored tatHSV-TK or tatHSV-TKΔ. All 20 colonies had the wild-type HSV-TK (Fig. 2.4). This corresponds to at least a 10,000-fold enrichment for AZT kinase activity.

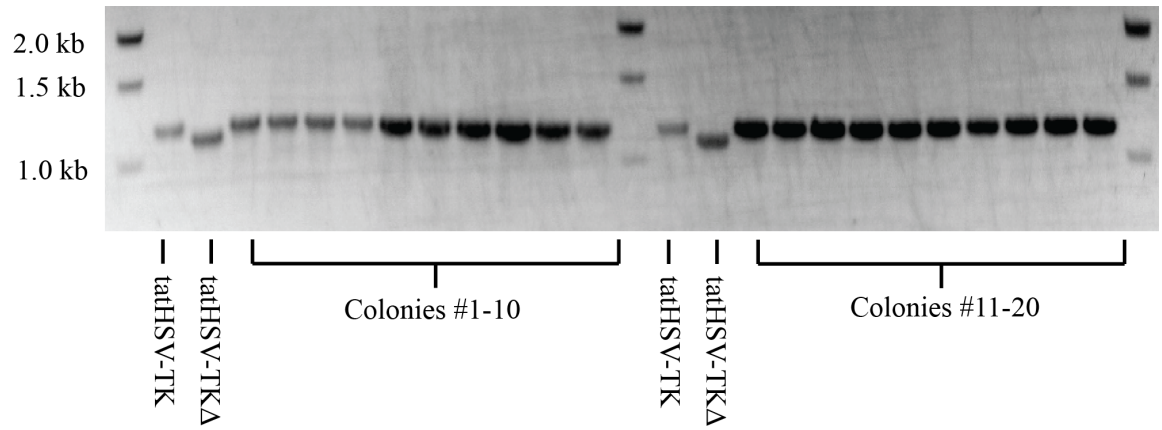

Figure 2.4: PCR assay demonstrating at least a 10,000-fold enrichment for active tatHSV-TK over an inactive tatHSV-TKΔ. BW14012 cells expressing one of the two proteins were mixed at a 1:10,000 ratio (tatHSV-TK:tatHSV-TKΔ) ratio. Twenty colonies formed when 500,000 CFUs (determined under non-selective conditions) were plated on 40 μg/ml AZT. PCR-amplified *hsvtk* genes from these 20 colonies indicated that all twenty contained tatHSV-TK. Expected band sizes of tatHSV-TK and tatHSV-TKΔ in this PCR assay are 1226 bp and 1160 bp, respectively.

Discussion:

This study establishes a new method for positive selection of nucleoside kinase activity in *E. coli*. The selection is capable of spatially separating the heterologous kinase's activity from endogenous kinases. The translocation of the kinase to the periplasm provides a way to test phosphorylation activity without interference from endogenous kinase activity and without disrupting internal cellular function. In the presence of periplasmic HSV-TK, an 80-fold increase in

resistance towards AZT was observed when compared to inactive HSV-TK. This resistance sharply declined beyond 40 μg/mL AZT. We propose that this viability threshold provides an opportunity to select for higher activity variants of engineered HSV-TK in directed evolution experiments. The high degree of enrichment seen during mock selections at 40 μg/ml AZT supports this proposal. However, the ability of the selection to distinguish HSV-TK variants with small improvements in activity has not been tested.

While HSV-TK is known for its high activity towards nucleoside analogs, the robustness of the selection should also permit less promiscuous nucleoside kinases, such as deoxycytidine kinase, to generate resistance towards other nucleoside analogs (26). In theory, this selection could be applicable to any nucleoside kinase that can be functionally expressed in the periplasm and can phosphorylate toxic nucleoside analogs. Additionally, a similar positive selection process can be envisioned for nucleoside phosphatases in which periplasmically-expressed nucleoside phosphatases would remove phosphates from essential nucleotides to allow them to be transported in the cytoplasm in nutrient deficient conditions. This would rescue the cell from nucleoside starvation.

The selection strategy should work for kinase activity on any nucleoside that is toxic to *E. coli*. To apply this selection to a nucleoside kinases one must first establish that the nucleoside is toxic to *E. coli*, which requires that it be transported into the cytoplasm. The outer membrane of *E. coli* is permeable to a vast variety of molecules through passive diffusion from outer membrane porins but its selectivity substantially increases at the inner membrane. Uncharged nucleosides are

transported to the cytoplasm via *nupC* and *nupG* (27). These two nucleoside permeases have also displayed the ability to translocate a number of nucleoside analogs (25, 27). After transport into the cytoplasm, the nucleoside must be toxic itself or metabolized into toxic compounds. This process could be facilitated by heterologously expressed nucleoside kinases, should endogenous, cytoplasmic kinases be unable to do this reaction.

The selection also requires that the nucleoside kinase to be subject to selection is capable of being exported to the periplasm in functional form. For HSV-TK, we found that the Tat pathway for periplasmic translocation of folded proteins performed best, but other kinases may work better with one of the other export pathways. Some nucleoside and nucleoside analogs conceivably could interact with proteins in the periplasm and this interaction may affect the ability of the kinase to phosphorylate the nucleoside to provide resistance. However, this is a low-probability event since nucleosides have no known function outside the cytoplasm in bacteria. Although *E. coli* scavenges inorganic phosphate from nucleotides using PhoA, it has been suggested that other putative phosphatases may also be able to fulfill this role under highly stressed conditions (28, 29). In such a scenario, these genes must also be knocked out for the selection to work properly. This scenario was not observed under the stress of AZT and would not be expected for other nucleoside analogs; scavenging phosphatases are regulated by the abundance of extracellular inorganic phosphates, a molecule that the selection media must provide in order for any engineered kinase to work (29).

The key advantage of the selection scheme in this study is in the kinase's localization. Previous positive selection methods have attempted to dampen the noise from endogenous enzymes and reduce disruptions to cell function by genetically knocking out certain bacterial kinases or inhibiting enzymes involved in nucleoside metabolism with small molecules (6, 30-32). These methods yielded a positive selection, but one with strict limitations. The selections were stifled by the use of the small molecule protein inhibitors, which can have some base-line toxicity, and perturbations to normal bacterial metabolism through gene deletions. In addition to the base toxicity, some inhibitors used to maintain selection fidelity were partially metabolized by the engineered enzyme (33). This resulted in a feedback loop that placed a cap on the level of kinase activity that could be selected. Engineered kinases with improved activity could metabolize the inhibitor at a higher rate, which would begin generating more toxicity. These factors convolute directed evolution experiments by increasing false positive rates and potentially killing the most effective variants. Most positive selections in these experiments needed multiple rounds of screening after selections to identify successfully engineered enzymes amongst the false positives. By localizing the kinase to the periplasm, the need to inhibit endogenous kinases through gene deletions and molecular inhibitors has been removed. The only required deletion is for phoA, a periplasmic enzyme that is not involved in metabolism inside the cell. This yields a selection that is free of metabolic stress from protein inhibitors and gene deletions.

We believe that the positive selection developed in this proof-of-principle study should be capable of identifying engineered nucleoside kinases and possibly

nucleoside phosphatases with increased specific activity or expression levels compared to wild-type. Discovery of more substrate-specific, highly stable and highly active variants of such enzymes has direct implications for biotechnology. These modified enzymes can also be used as reporter genes for experiments or potentially be utilized in GDEPT experiments. Further work will need to be done to examine if this selection has a bias towards selecting for stability (i.e. increased protein abundance) or specific catalytic activity of kinases.

Acknowledgements:

We thank Margaret Black for providing vectors containing the *hsvtk* gene.

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Chapter 3: Development of a cancer-marker activated enzymatic switch from the herpes simplex virus thymidine kinase

Summary:

Discovery of new cancer biomarkers and advances in targeted gene delivery mechanisms have made gene-directed enzyme prodrug therapy (GDEPT) an attractive method for treating cancer. Recent focus has been placed on increasing target specificity of gene delivery systems and reducing toxicity in non-cancer cells in order to make GDEPT viable. To help address this challenge, we have developed an enzymatic switch that confers higher prodrug toxicity in the presence of a cancer marker. The enzymatic switch was derived from the herpes simplex virus thymidine kinase (HSV-TK) fused to the CH1 domain of the p300 protein. The CH1 domain binds to the C-terminal transactivation domain (C-TAD) of the cancer marker hypoxia inducible factor 1α (HIF1-α). The switch was developed using a directed evolution approach that evaluated a large library of HSV-TK/CH1 fusions using a negative selection for azidothymidine (AZT) toxicity and a positive selection for dT phosphorylation. The identified switch, dubbed TICKLE (Trigger-Induced Cell-Killing Lethal Enzyme), confers a 4-fold increase in AZT toxicity in the presence of C-TAD. The broad substrate specificity exhibited by HSV-TK makes TICKLE an appealing prospect for testing in medical imaging and cancer therapy while establishing a foundation for further engineering of nucleoside kinase protein switches.

Introduction:

The variety of approaches for treating cancer include those that target different aspects of the disease. These approaches range from small molecule drugs that disrupt internal cancer cell replication to protein therapeutics, such as antibodies designed to specifically inhibit cancer-proliferating growth factor receptors [1–3]. While many of these therapeutic approaches show an ability to reduce tumor size and impede the spread of cancer, they also can subject the patient to harsh side effects. Amongst the most effective and recognized categories of cancer treatment is chemotherapy, which is well known for side effects that include hair loss, severe fatigue, nausea and loss of appetite [4]. These side effects arise from cancer drugs killing normal, healthy cells along with cancer cells [1,2,4]. The off-target activity of these drugs limits the dosage that can be administered to patients [2,5].

A number of attempts have been made to increase specificity of cancer drugs towards cancer cells and reduce their toxicity towards healthy cells. This has led to the development of prodrugs, chemically-inactivated drugs that depend on cellular metabolism to be reactivated and generate toxicity [6,7]. Such drugs aim to increase specificity towards cancer cells by relying on metabolic rate discrepancies between the cancer cells and regular cells; the typically high rate of metabolism within cancer cells generates more toxicity from prodrugs at lower doses compared to healthy cells [8]. Since this approach still allows prodrug activation in healthy cells, a modified form of the therapy called gene-directed enzyme prodrug therapy (GDEPT) was developed [9]. GDEPT increases specificity towards cancer by

delivering a non-native gene responsible for activating prodrugs into cancer cells [10]. In theory, the targeted delivery allows only cancer cells to harbor the gene and its corresponding protein that is capable of activating the prodrug. However, most delivery systems lack the required target specificity to make GDEPT a viable approach for cancer treatment [8,11]. This lack of effective delivery systems could be addressed by engineering the non-native gene product to have its activity dependent on specific protein markers of cancer through the appropriate fusion of the enzyme to a protein that binds the cancer marker. Our lab has previously developed such a protein, dubbed HAPS59, by fusing the yeast cytosine deaminase (yCD) to a CH1 domain. The binding of the cancer marker, HIF1-α, to the CH1 domain increases the cellular accumulation of HAPS59, causing increased activation of the prodrug 5-fluorocytosine (5FC) [12]. This engineered "enzymatic switch" can potentially provide the lacking cancer specificity by only activating the prodrug in the presence of specific cancer markers.

Multiple studies have shown that combining GDEPT systems can have synergistic effects in generating toxicity towards cancer [13,14]. While many factors contribute to this observation, the bystander effect displayed in GDEPT systems can be tremendously useful in killing cancer cells. Combining enzyme-prodrug combinations may allow for proper attenuation of this effect without having to change dosage of each drug [9,15]. The enzyme-prodrug combination of cytosine deaminase (CD) and 5-FC is known for having a large bystander effect, whereas the combination of the herpes simplex virus thymidine kinase (HSV-TK) and ganciclovir (GCV) are known for its smaller bystander effect [15]. When the two enzyme-

prodrug systems are used together, a large synergistic effect is seen [13]. For this reason, the prospect of creating an enzymatic switch out of HSV-TK that can be used alongside previously developed enzymatic switches from yCD is appealing [12,16]. Having two switches with increased target specificity could allow for a synergistic approach to fighting cancer at even higher dosages. Furthermore, HSV-TK's broad substrate specificity lends itself to be used as a reporter gene with other nucleoside analogs besides GCV, and a switch derived from the enzyme could have important implications in medical imaging [17,18]

An established strategy for developing enzymatic switches involves fusing a signal-recognizing domain to an output domain [19]. Conceptually, the fusion of the two can couple signal recognition to a specific output. However, the difficulty in implementation of this strategy revolves around identifying the site of insertion of the signal-recognition domain that results in the switch property. A directed evolution approach involving the subjection of a library of variants with the signal-recognition domain in each insertion point on the output domain to selection pressures is useful [20]. The selection would identify specific switches without the need to predict or understand the mechanism by which the switch functions.

In this study, we used a directed evolution method to engineer the HSV-TK enzyme to have its cellular activity modulated by the presence or absence of the C-terminal transactivation domain (C-TAD) of the hypoxia-inducible factor 1-alpha (HIF1-α) cancer marker. HSV-TK possesses a large active site and is promiscuous in phosphorylating a variety of different nucleoside analogs [21,22]. HIF1-α is a

transcription factor that is upregulated in hypoxic environments, such as tumors, but is almost non-existent in normoxic cells [23]. The transcription factor is involved in regulating cell proliferation, apoptosis and tumor angiogenesis in hypoxic conditions [24]. Sensitizing HSV-TK activity to respond to the presence of HIF1-α would provide a way to generate more cancer cell-specific toxicity. To create the switch, the CH1 domain of the p300 protein was inserted into the amino acid backbone of HSV-TK. In its native context, the C-TAD domain of HIF1-α interacts with the CH1 domain and the CH1 domain goes from a molten globule state to a structured state upon binding of the C-TAD [25,26]. Our aim was to exploit this interaction and have it influence the level of kinase activity in an HSV-TK/CH1 fusion protein. The directed evolution method we utilized involved a two-tiered selection scheme to sequentially select for an "On" and an "Off" state of a switch. This strategy led to the discovery of a protein switch we dubbed TICKLE (Trigger-Induced Cell Killing Lethal Enzyme) that confers C-TAD dependent azidothymidine (AZT) toxicity to *E. coli* cells. TICKLE establishes a way to modulate cellular activity of HSV-TK at the protein-level and provides a platform to further engineer kinase-based protein switches tailored towards specific substrates.

Materials & Methods:

Unless otherwise specified, all molecular biology protocols were performed using New England Biolabs' (NEB) High-Fidelity Phusion Master Mix for PCR, Invitrogen's Gel Purification Kit for gel extraction and Zymo's DNA Clean & Concentrator kit for DNA purification (5 μg loading capacity). All DNA

phosphorylation reactions were conducted using NEB's T4 Polynucleotide Kinase with corresponding buffer.

Vectors & Strains:

The plasmid pMCC contains a wild-type *hsv-tk* gene and was a gift from Dr. Margaret Black (University of Washington). The *hsv-tk* gene on the vector is under the control of the temperature sensitive lambda pR promoter system and confers ampicillin resstance (AmpR). We created a previously described inactive variant of the *hsv-tk* gene as a control [27]. The pGA-GSTHIF1α vector housing the C-terminal transactivation domain (C-TAD) of HIF1-α attached to a GST-tag on its N-terminus was previously described, as was a corresponding control vector with GST only called pGA-GST [16]. Both GST and GSTHIF1α genes were amplified using PCR and cloned into a spectinomycin resistance (SpecR) pSkunk2 vector (pSK2) under the *tac* promoter [16]. Unless otherwise indicted, all experiments were performed using the pMCC vector for expression of HSV-TK, the CH1/HSV-TK libraries and TICKLE while the pSK2 vector was used for GST or C-TAD-GST. Vectors utilized in the creation of genomic deletions (pKD4, pKD46, pRHAM) have been described previously [28].

E. coli KY895 (-*tdk*) was acquired from Coli Genetic Stock Center at Yale University. *E. coli* 5α was used for cloning and purchased from NEB. *E. coli* NS01 (-*tdk, -thyA*) was a strain created using the Lambda Red Recombinase system and *E. coli* KY895 as a parent strain [29]. The strain has its chromosomal *thyA* gene replaced with a *nptII* gene to confer kanamycin resistance and a copy of *thyA* is provided on the pRHAM vector under the control of a rhamnose the promoter. *E.*

coli. NS01 is conditional viable in the presence of rhamnose and non-viable in the presence of glucose and absence of rhamnose.

Linearized HSV-TK Insertion Library:

We utilized inverse PCR to develop a linearized DNA library with the *hsv-tk* gene opened up after 273 separate codons as described [30]. Separate PCR reactions were performed using primer pairs targeting 273 different insertion sites in a 96 well format to get a linearized library of pMCC-*hsvtk*. The library was named "linear pNYS". Samples from every PCR were electrophoresed on 1.2% Tris-actetate-EDTA gels to confirm the correct band size. Pooled PCR reactions were electrophoresed on a TAE gel and the desired band extracted from the gel. The resulting elution was purified further using Zymo Research's DNA Clean & Concentrator (25 μg loading capacity).

Degenerate & specific CH1 linkers:

To create a degenerate CH1 linker library, we ordered oligonucleotides consisting of 0,1,2 and 3 degenerate NNK codons from Integrated DNA Technologies. The oligonucleotides were designed to bind to the 5'- and 3'- termini in the CH1 gene on the plasmid pRW0017 with the degenerate codons on the flanks [16]. We then created the DNA library by PCR of CH1 using these degenerate primers. The PCR product was extracted from the gel and purified with Zymo Research's DNA Clean & Concentrator (5 μg loading capacity). The degenerate PCR library was dubbed "x(XXX)-CH1-x(XXX)". We also used PCR to create CH1 constructs with predefined flanks composed of every permutation of GGS, $(GGS)_2$,

$(GGS)_3$ on the N-terminus and every permutation of STT, $(STT)_2$, $(STT)_3$ on the C-terminus. We named this PCR library x(GGS)-CH1-x(STT). PCR was also used to amplify CH1 without added linkers for the purpose of a no-linker library.

HSV-TK – CH1 fusion & transformation:

To create library pNYS01, we phosphorylated the amplified CH1 without any linkers and ligated it with 1 μg of linear pNYS in a 3:1 molecular ratio (CH1 to pNYS01). The pNYS02 and pNYS03 libraries were created analogously using x(GGS)-CH1-x(STT) and x(XXX)-CH1-x(XXX) as the inserts, respectively. The ligation reactions were conducted overnight at 16°C and purified after. The purified ligation was then transformed into NEB's 5α electrocompetent cells. Transformed cells were grown in liquid media at 30°C for one hour and then plated on large tryptone agar plates. Colonies were grown overnight at 37°C and then collected in liquid tryptone media with 15% glycerol. Collected stocks were frozen at -80°C for future use in selections while a portion was extracted for plasmid DNA of pNS01 using a miniprep kit.

One hundred nanograms of extracted, circular pNYS01, pNYS02 or pNYS03 were transformed into *E. coli* KY895 electrocompetent cells for use in selections. Transformed cells were incubated for one hour and plated at 37°C overnight on a large tryptone agar plate. Cells were collected and frozen for future use.

Two-tiered selection & screening:

We first subjected the libraries to a negative selection to select for the "Off" state of the enzymatic switch. Bacteria were plated on solid tryptone media with 10

μg/mL AZT and 25 μg/mL Amp. The plates were incubated at 37°C overnight and the surviving colonies were collected in liquid media. After colony collection, the plasmid DNA was then extracted from the collected stock and 100 ng of the DNA was transformed into *E. coli* KY895 strain with pSK2-GSTHIF1α. These transformants were used in a positive selection to select for the "On" state of the enzymatic switch. This specific selection protocol was adopted from a previous study selecting for higher activity HSV-TK variants [31]. In this selection, bacteria were plated on tryptone media with 10 μg/mL 5-Fluorouridine (5-FdU), 2.5 μg/mL uridine (dU), 10 μg/mL thymidine (dT), 25 μg/mL Amp and 25 μg/mL spectinomycin (Spec). The plates were incubated at 37°C for 28 hours. After the selection, we picked the surviving colonies and grew them overnight at 37°C in tryptone media with 0.2% glucose (Glu) so that they could be screened for switching activity.

We used a liquid culture spotting assay to screen for switching activity. The overnight cultures from the positive selection were normalized to an optical density (OD) of 0.6 and then diluted 10,000 fold. 1.5 μl of the diluted cultures were then spotted on two different types of solid tryptone media screening plates: the first screening plate tested for "On" activity and had 10 μg/mL AZT with 0.3 mM IPTG while the second plate tested for "Off" activity and had 10 μg/mL AZT with 2% Glu. Plates were incubated at 37°C overnight for 18 hours and then compared to qualitatively assess cell density of corresponding spots between the "On" and "Off" plate. We selected candidates that displayed a stark difference in cell density and prepared overnight cultures at 37°C in liquid media. The cultures were extracted for

plasmid DNA, transformed into a fresh cellular background (*E. coli* KY895 with pSK2-GSTHIF1α) and analyzed again in the same liquid culture spotting assay to confirm the switching phenotype.

AZT toxicity assay

We conducted a liquid cell viability assay to assess the degree of switching between "On" and "Off" states of the HSV-TK switches. One million CFUs from overnight cultures of *E. coli* KY895 containing the switch candidate and pSK2-GST or pSK2-GSTHIF1α were added to liquid LB media containing 0.2% Glu, 25 μg/mL of Amp, 25 μg/mL Spec and different concentrations of AZT. Arabinose (0.01%) was added to wells containing cells with pSK2-C-TAD-GST and pSK2-GST. A positive control of cells with wild-type HSV-TK and a negative control of cells with knocked out HSV-TK (HSV-TKΔ) were also grown in identical fashion and used in the cell toxicity assay in the same liquid media conditions, excluding the addition of arabinose and Amp. After six hours of incubation at 37°C we measured the optical density of the cultures at 600 nm (OD_{600}).

Activity towards dT:

Switch candidates were transformed into this *E. coli* NS01 housing pSK2-GST or pSK2-GSTHIF1α for a liquid cell viability assay. In 96 well culture plates, 500,000 CFUs of each strain type were inoculated into tryptone media containing 0.2% Glu, 5 μg/mL chloramphenicol (Cm), 0.01% arabinose, 25 μg/mL Amp, 25 μg/mL Spec and different concentrations of dT. After eight hours of incubation at 37°C we measured the OD_{600}.

Western Blot analysis:

We expressed TICKLE with and without the C-TAD in *E. coli* overnight and normalized the OD between the two cultures the next day. Additional cultures included cells expressing HSV-TK and cells expressing neither HSV-TK nor TICKLE. The cultures with an equal number of cells were then lysed using Novagen's BugBuster reagent as per the supplied protocol. The soluble fractions were collected after chemical lysis of the bacterial cultures and then electophoresed on a NuPage 4-12% Bis-Tris protein gel (ThermoFisher Scientific) using SDS-PAGE. The proteins from the gel were then transferred on to a polyvinylidene fluoride (PVDF) membrane using Biorad's Trans-Blot SD Semi-dry Transfer Cell for 15 minutes at 15V. After the transfer, the primary antibody against HSV-TK (Santa Cruz Biotechnologies, Inc.) was incubated with the membrane in Tris-buffered saline-Tween (TBST) buffer at a 3:500 antibody:buffer ratio for 1 hr at 4°C. The anti-goat secondary antibody (Biorad Laboratories, Inc) was loaded on to the western blot in TBST buffer at a 3:500 antibody:buffer ratio. No wash steps or blocking steps were performed. We visualized the western by adding HRP substrate using Biorad's Clarity Western ECL Substrate as per the protocol provided. The chemiluminescence was observed using Biorad's Universal Hood II and Quantity One Software.

Results

Library construction

To sensitize the HSV-TK enzyme to the C-TAD of HIF1-α, the CH1 domain needed to be incorporated into the kinase. We generated DNA libraries of HSV-

TK/CH1 fusions in which the CH1 domain was incorporated at different insertion sites in HSV-TK using different types of linkers. These libraries were created by blunt-end ligation of the CH1-encoding DNA in between codons of the *hsvtk* gene (Fig. 3.1). We developed three libraries. The first library was dubbed pNYS01 and had the CH1 domain directly incorporated at 273 different insertion points in HSV-TK (Table 3.1, end of chapter). We only chose 273 of the 376 possible sites for domain insertion after scrutinizing the protein structure for solvent accessible regions and avoiding regions deeply embedded inside the protein. A second library, pNYS02, was created with every permutation of (GGS), $(GGS)_2$, and $(GGS)_3$ linkers on the N-terminus of the CH1 and every permutation of (STT), $(STT)_2$, and $(STT)_3$ linkers on the C-terminus. The CH1 domain, with these linkers, was incorporated into the same 273 insertion points of HSV-TK. We also constructed a third library, pNYS03, which also used the same 273 insertion points as the other two libraries but had the CH1 domain connected to HSV-TK through all permutations of (XXX), $(XXX)_2$, and $(XXX)_3$ on both the N- and C- termini of the CH1 domain. X designates any amino acid and was encoded by NNK degenerate codons.

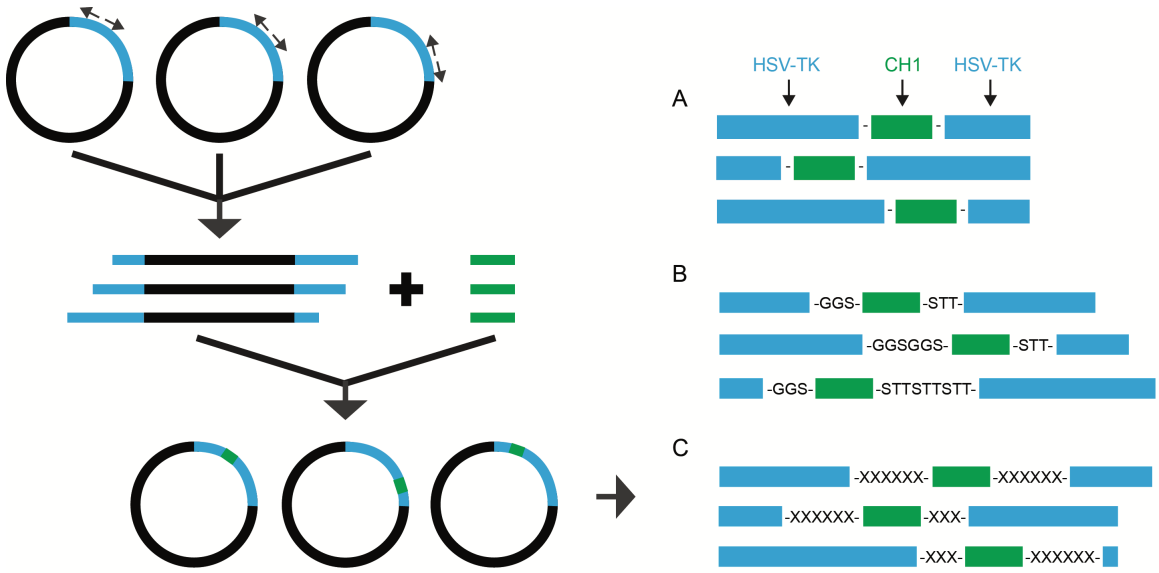

Figure 3.1: Construction of libraries.

Libraries with the CH1 domain (green) connected to HSV-TK (blue) were created using inverse PCR in which the plasmid containing *hsv-tk* was linearized at specific codons within the gene. The linearized plasmid was ligated with CH1 DNA to create the circularized plasmid library with the CH1 inserted into HSV-TK at different sites. Various linkers were added to the CH1 DNA by PCR before the ligation step to generate different types of libraries such as a) pNYS01, b) pNYS02 and c) pNYS03.

The pNYS01 library was constructed to test and identify variants that could function as a switch without the added flexibility of linkers. Since the library had the CH1 inserted into 273 sites of HSV-TK, the theoretical library size was only 2 x 273 = 546 (accounting for forward and backward insertions). The pNYS02 library was the first library to possess variants with linkers connecting the CH1 to HSV-TK. The Gly-Gly-Ser linker used for the N-terminus of CH1 is commonly utilized to generate flexibility between domains. Using Ser-Thr-Thr linker for the C-terminal linker of CH1 was a mistake that resulted from incorrect primer design for oligonucleotides used in PCRs to amplify the CH1 encoding DNA. We decided to

move forward with the library despite the mistake, which turned out to be fortuitous, as will be shown. The pNYS02 library had a theoretical size of 4914 members since the CH1 construct could be incorporated into each of the 273 insertion point with 9 possible permutations of linker combinations. The third library, pNYS03, was a very large library that was constructed to test 1, 2, and 3 amino acid linkers of variable compositions connecting the CH1 to HSV-TK. The theoretical library size was approximately 141.6 million members.

The pNYS01 and pNYS02 libraries were comprised of about two hundred thousand transformants each. Considering that their theoretical library size was 546 and 4914 variants, respectively, the probability that each of these libraries is complete is essentially 100% [32]. The pNYS03 library consisted of 11 million transformants for testing, which is only a fraction of the possible number of different sequences.

Selections and screens identify a switch

We subjected all libraries to a two-tiered selection designed to identify HSV-TK/CH1 fusion proteins that had their cellular kinase activity increase upon the expression of the C-TAD domain of HIF1α. (Fig. 3.2) [25]. An initial negative selection in *E. coli* KY895 cells identified variants that lacked AZT phosphorylation activity in the absence of C-TAD expression. *E. coli* KY895 cells lack endogenous thymidine kinase (Tdk) activity and cannot metabolize AZT into its toxic form. However, library variants capable of phosphorylating AZT into its monophosphate form, AZT-MP, would be able to kill their host cells from AZT-MP toxicity. We subjected surviving members of this negative selection to the subsequent positive

selection to identify variants that could phosphorylate deoxythymidine into dTMP in presence of the C-TAD of HIF1α. All endogenous means of dTMP production were inhibited through the addition of 5-FdU. There are two enzymes in *E. coli* capable of producing dTMP, thymidine kinase (Tdk) and thymidylate synthase (ThyA) [33]. As mentioned above, *E. coli* KY895 does not possess Tdk and 5-FdU directly inhibits thyA. Thus, growth depends on the ability of the HSV-TK/CH1 fusions to produce dTMP.

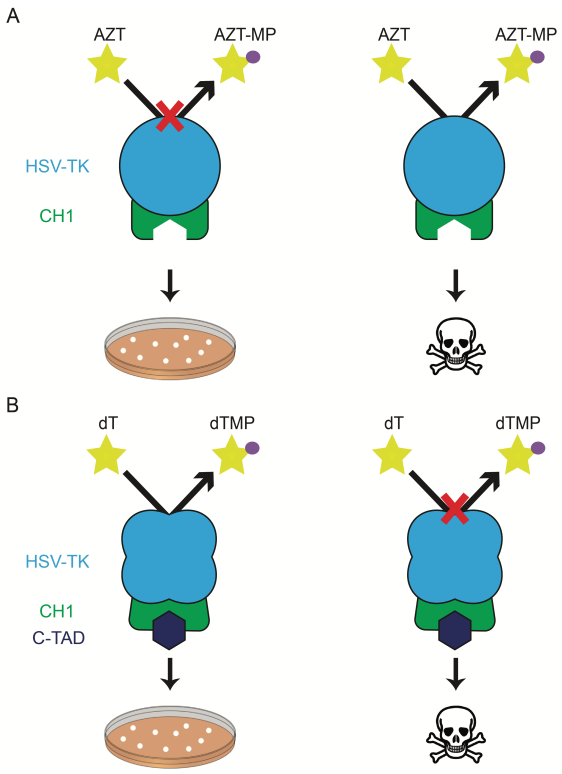

Figure 3.2: Genetic selections.

A) The negative selection in *E. coli* KY895 identifies variants that lack kinase activity in the absence of the C-TAD of HIF1-α. Variants that possess activity in this condition produce toxic AZT-MP while variants lacking kinase activity do not and thus survive. B) The positive selection, also done in *E. coli* KY895, selects for variants with the ability to phosphorylate dT to dTMP in the presence of the C-TAD. Cells that are unable metabolize the necessary dTMP are killed.

Approximately 10,000 colony forming units (CFUs) of cells harboring pNYS01 and pNYS02 and 10^6 CFUs of cells harboring pNYS03 were plated on negative selection plates. Our negative selections displayed a cell survival rate of about 70%. The high survival rate in the negative selection is not surprising, since we expected that most insertions of CH1 into HSV-TK would compromise enzyme activity. In the subsequent positive selection in the presence of coexpressed C-TAD, 5000 CFUs/plate of the pNYS01 and pNYS02 libraries were plated whereas 500,000 CFUs/plate were spread on positive selection plates for the pNYS03 library. These selections resulted in 64 colonies from the pNYS01 library (on one plate), approximately 300 colonies for the pNYS02 library (on two plates) and approximately 3600 colonies from the pNYS03 library (on four plates). Over 2400 hits from the positive selections of pNYS01, pNYS02, pNYS03 were screened for a switching phenotype. We screened all hits from the pNYS01 library and approximately 60% of the hits from the pNYS02 and pNYS03 libraries by a liquid culture spotting assay where cells from the positive selection expressing the variant were spotted on negative selection plates with and without C-TAD being expressed (C-TAD expression was induced by the addition of arabinose to the plate). Plasmids from hits in this screen were retransformed into fresh KY895 cells and retested by the spotting assay.

While no protein switches were found in pNYS01, one HSV-TK/CH1 fusion from each of the pNYS02 and pNYS03 libraries reproducibly showed increase AZT susceptibility with coexpression of C-TAD of HIF1α. Both constructs shared the same amino acid sequence (Fig. 3.3a) but differed at the nucleotide level. Based on

DNA sequencing, the CH1 domain is inserted after the 150th amino acid of HSV-TK (Fig. 3.3a). This insertion site is part of an unstructured loop in HSV-TK (Fig 3.3b). The N-terminus of the CH1 was connected to the HSV-TK enzyme through a Gly-Gly-Ser linker while the C-terminus was connected using a Ser-Thr-Thr linker. We were struck and encouraged by the fact that both the pNYS02 and pNYS03 libraries resulted in selection of the same protein and that the selections performed on the third library (with randomized linkers) resulted in selection of the same STT C-terminal linker that was an "error" in the design of the second library. We designated this switch TICKLE for "Trigger Induced Cell Killing Lethal-Enzyme".

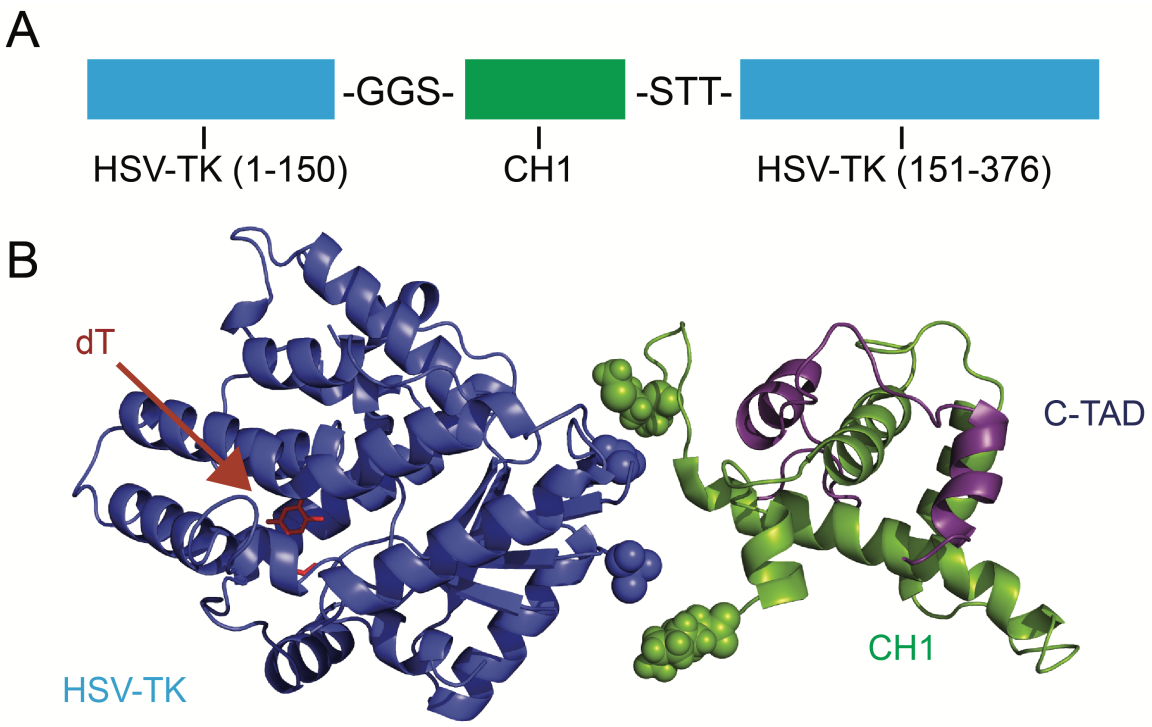

Figure 3.3: TICKLE switch

A) Sequence of TICKLE. TICKLE is comprised of a CH1 domain insert in between the 150th and 151st residues of HSV-TK. The N-terminus of the CH1 is connected to HSV-TK by a Gly-Gly-Ser linker and the C-terminus is connected by a Ser-Thr-Thr linker. B) Structure models of HSV-TK (blue, PDB #1KIM [46]) adjacent to CH1 (green) with the C-TAD of HIF1-α (violet) bound (PDB #1LE3 [25]). Space-filled spheres indicate the sites of fusion (i.e. insertion) of CH1 into HSV-TK. The insertion site in HSV-TK (between residues 150 and 151) is within an unstructured loop (residues 148-153) in the crystal structure, thus residues 147 and 154 are indicated instead. Thymidine (dT, red) is shown in the active site of HSV-TK.

TICKLE switch renders cells AZT sensitive in a C-TAD dependent manner

AZT toxicity assays were conducted in solid and liquid media to determine the extent to which expression of C-TAD of HIF1-α caused cells expressing TICKLE to be sensitive to AZT. As we expressed the C-TAD domain as a fusion with glutathione S-transferase (GST), we expressed GST alone as the negative control. In

the spot assay, serial dilutions of cells were challenged to grow in the presence of AZT. For cells expressing TICKLE, co-expression of C-TAD-GST made the cells more susceptible to AZT compared to co-expression of GST (Fig 3.4a). The liquid assay evaluated the relative growth of *E. coli* KY895 containing TICKLE by optical density, in the presence of different levels of AZT. The presence of C-TAD-GST caused a 4-fold increased in AZT toxicity relative to GST (Fig. 3.4b). Regardless of whether C-TAD is expressed, TICKLE is inferior to HSV-TK at making the cells susceptible to AZT (Fig 3.4b). Thus, the insertion of the CH1 domain as found in TICKLE, greatly compromises the ability of the HSV-TK to confer AZT sensitivity to cells, but this toxicity can be partially restored to higher levels by the presence of C-TAD. Presumably this increased AZT toxicity results from the C-TAD domain binding to the CH1 domain of TICKLE and either increasing its specific enzyme activity or increasing its cellular abundance.

We also examined the importance of the STT sequence in the C-terminal linker of TICKLE by mutating it back to the intended GGS linker of the second library (dubbed TICKLE (S243G, T244G, T245S)) as well as mutating it to SST (dubbed TICKLE (T244S)). AZT toxicity assays showed that the STT linker was important if not vital to TICKLE's switch function. TICKLE (T244S) caused only a 2-fold switching effect, compared to the 4-fold effect seen in wild-type TICKLE (Fig 3.4c). The T244S mutation decreased switching by increasing AZT toxicity in the absence of C-TAD. Replacing the STT linker with the intended GGS linker not only abolished switching, but also eliminated TICKLE's ability to make the cells sensitive to AZT (Fig. 4c).

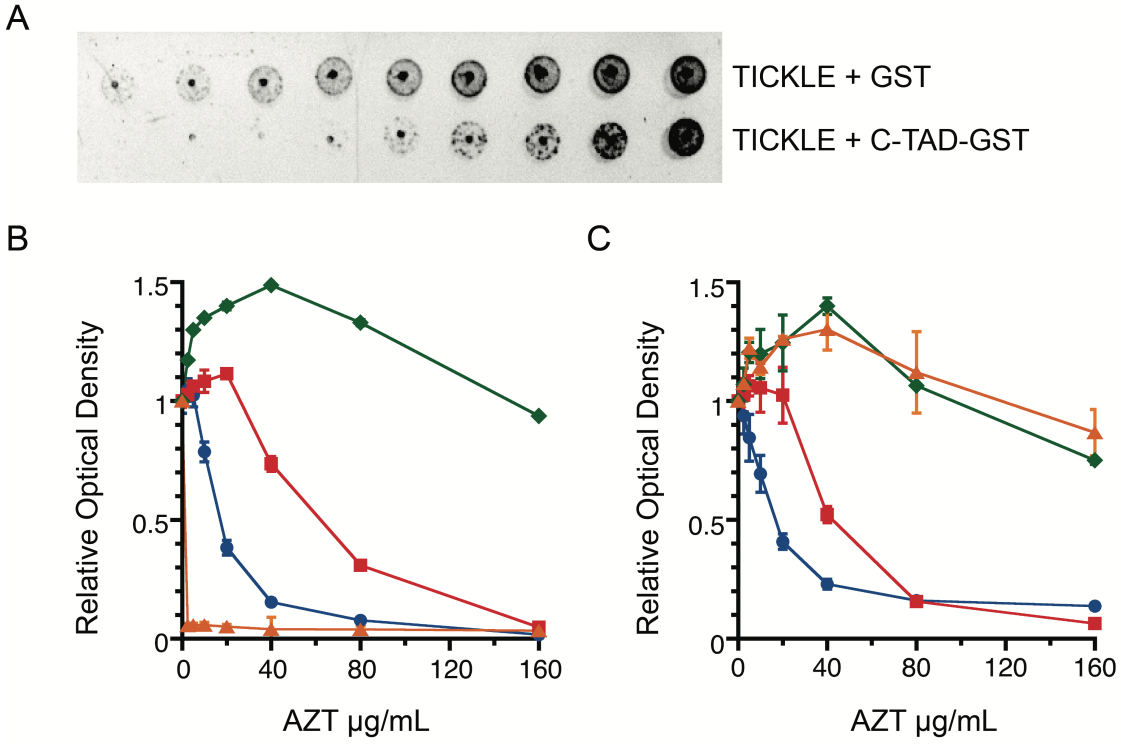

Figure 3.4: AZT toxicity assay

A) C-TAD coexpression increases the AZT toxicity of *E. coli* KY895 cells expressing TICKLE. Equal number of cells expressing TICKLE + GST or TICKLE + C-TAD-GST were spotted on negative selection plates containing 10 µg/mL AZT and incubated 18 hours at 37 °C. The different spots represent a series of 1:2 serial dilutions of the culture, with the highest cell concentration at the right.B) *E. coli* KY895 cells expressing the indicated proteins were challenged to grow in liquid media for six hours in the presence of different concentrations of AZT. The relative OD_{600} normalizes the OD_{600} to that in the absence of AZT. Blue circles, TICKLE + C-TAD-GST; red squares, TICKLE + GST; orange triangles, HSV-TK (positive control); and green diamonds, inactive HSV-TK (negative control). C) Blue circles, TICKLE (T244S) + C-TAD-GST; red squares, TICKLE (T244S) + GST; orange, TICKLE (S243G, T244G, T245S) + GST; green diamonds, TICKLE(S243G, T244G, T245S)+ C-TAD-GST.

TICKLE switch requires C-TAD coexpression for rescuing cells deficient in dTMP production

To further confirm TICKLE functioned as a switch we tested its ability to rescue growth of cells deficient in dTMP production. dTMP is essential to cell survival. A conditionally lethal cell strain (NS01) was created from the *tdk* knock out strain KY895. NS01 has *thyA* deleted from the chromosome and a plasmid containing *thyA* under the control of the rhamnose-inducible promoter system. In the presence of glucose (and absence of rhamnose), the inability of NS01 to produce sufficient dTMP makes the strain non-viable, but growth can be rescued by the addition of rhamnose (Fig 3.5a). In the absence of rhamnose, growth could be rescued by expressing HSV-TK (Fig 3.5b).

We utilized this strain in a growth assay that tested TICKLE's ability to generate dTMP in a C-TAD dependent manner. NS01 cells expressing TICKLE and C-TAD-GST or GST were challenged to grow under non-permissive conditions at different concentrations of dT. TICKLE was expressed from the pMCC plasmid while C-TAD-GST and GST were expressed from pSkunk2. TICKLE allowed the cells to grow, but only if C-TAD was expressed (Fig. 3.5b). Cells not expressing the C-TAD showed a consistently low optical density regardless of dT concentration.

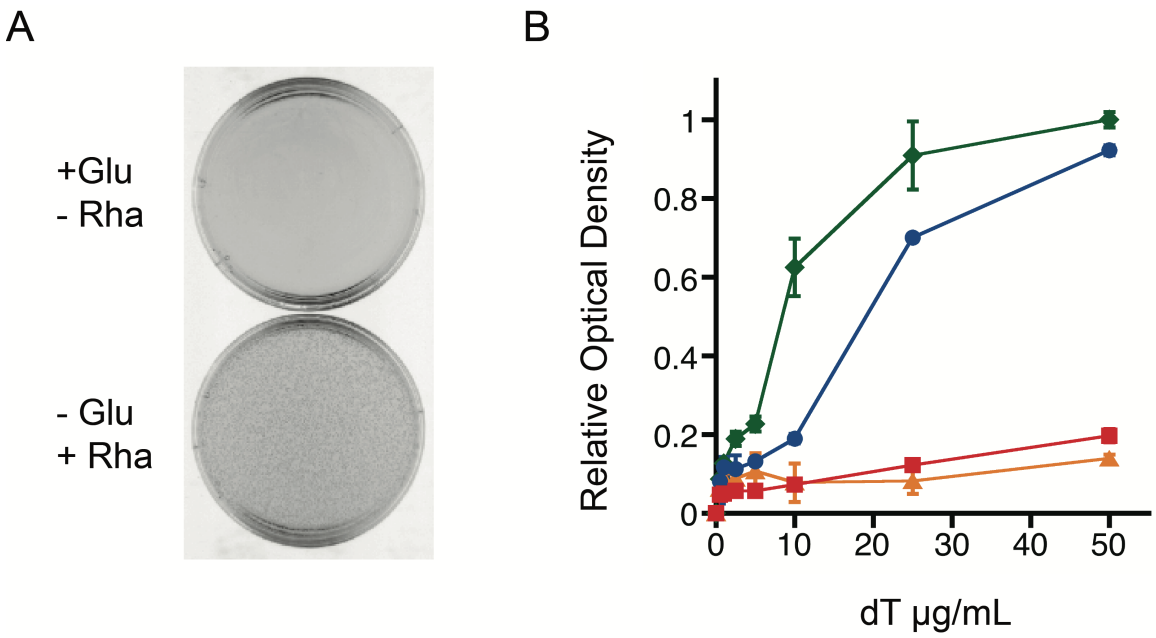

Figure 3.5: dT Growth Assay

A) *E. coli* NS01 is a conditional lethal strain that requires rhamnose for growth due to a deficiency in dTMP production. One thousand CFU cells were plated on tryptone media plates with 25 µg/mL kanamycin and containing 0.2% glucose (Glu) (top; non-permissive condition) or 0.2% rhamnose (Rha) (bottom; permissive condition) and incubated 18 hours at 37°C. B) *E. coli* NS01 expressing the following proteins were grown in non-permissive liquid media for eight hours in the presence of different concentrations of dT. Blue circles, TICKLE + C-TAD-GST; red squares, TICKLE + GST; orange triangles, inactive HSV-TK (negative control); and green diamonds, wild-type HSV-TK (positive control). The relative OD_{600} was normalized to the OD_{600} of the positive control at 50 µg/mL dT.

C-TAD increases TICKLE cellular abundance

Immunoblotting was used to better understand the switching mechanism behind TICKLE causing increased AZT toxicity in the presence of the C-TAD. One possibility is that C-TAD binding to TICKLE increases TICKLE's cellular abundance, thus increasing the total AZT kinase activity in the cell. Such a mechanism has been observed with previous switches built by domain insertion [12,34]and can result

from the binding event increasing the thermodynamic or proteolytic stability of the switch [35]. Cultures of *E. coli* KY895 expressing TICKLE with and without C-TAD co-expression were incubated overnight. We lysed an equal number of cells from the cultures (based on OD) and examined the soluble fraction of the lysate by western blot using anti-HSV-TK antibodies (Fig. 3.6). As in previous studies [36,37], HSV-TK expression in *E. coli* resulted in a multiple bands, the smaller of which are presumably proteolytic products. Expression of TICKLE resulted in higher weight bands as expected by the addition of the CH1 domain, but the intensity of the higher weight bands (especially the largest size band at about 53 kDa, the expected size of TICKLE) was much higher when C-TAD was coexpressed.

These results are consistent with the following model of TICKLE's mechanism. In the absence of C-TAD, TICKLE is an unstable protein and is rapidly degraded. In the presence of C-TAD, its binding to the CH1 domain of TICKLE decreases the rate of TICKLE's turnover in the cell, causing a higher level of cellular HSV-TK activity. The presence of bands smaller than TICKLE but larger than those of HSV-TK suggest a reason for why TICKLE is not a better switch than it is. These proteolytic products may lack the ability to bind CH1 but retain HSV-TK enzyme activity that would be unregulated by C-TAD.

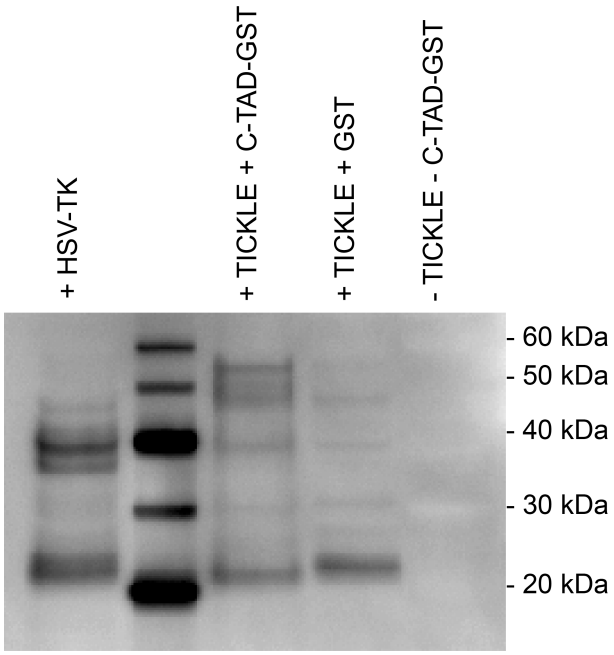

Figure 3.6: Relative accumulation of TICKLE as a function of C-TAD coexpression.

A western blot using anti-HSV-TK antibodies was performed on an SDS-PAGE gel of the soluble fraction from an equal number cells KY895 cells expressing the indicated proteins. Lane 1, HSV-TK; lane 3, TICKLE + C-TAD-GST; lane 4, TICKLE + GST, lane 5, no heterologous protein expressed. Lane 2 is the molecular weight marker.

Discussion

The development of TICKLE as an enzymatic switch establishes a way to modulate the activity of HSV-TK through the C-TAD of HIF1-α. Non-target toxicity during cancer therapy is a major obstacle in increasing dosage levels of drug. TICKLE's net level increases in the cell when in the presence of the C-TAD of a cancer marker, potentially providing a way to selectively activate prodrugs in cancer cells. The "Off" state of the enzymatic switch diminished levels of the enzyme when compared to the "On" state. The 4-fold increase in toxicity that is generated by the presence of the C-TAD is believed to be due to a stabilizing effect that occurs in

the presence of the C-TAD that leads to greater accumulation of the enzyme. Studies have shown that the CH1 domain exists in a molten globule state and transitions into a structured state after binding to the C-TAD domain [25]. This molten globule state of CH1 may cause TICKLE to be cellularly unstable, leading to increased degradation by proteases and lower net cellular activity towards AZT in bacteria. The binding of the C-TAD would stabilize the CH1 domain and TICKLE, which would lead to higher accumulation of this protein in the cell. This mechanism is supported by western blot analysis of TICKLE.

The data on C-TAD dependent accumulation does not rule out the possibility that the protein switch is functioning through an allosteric mechanism, as the mechanisms are not mutually exclusive. HSV-TK possesses five parallel β-sheets (known as the "CORE domain" in other kinases) which are collectively thought to function as a stable "anchor" that helps facilitate movements in the nucleoside binding domain and ATP-binding domain for catalysis [38]. The CH1 insertion is 6 amino acids from the β3 strand, which runs from L157 to D162 and is directly followed by a key active site residue (R163). This suggests that CH1 could destabilize the stable anchor region, disrupt domain mobility and reduce substrate phosphorylation. If the allosteric mechanism is in effect, the binding of the C-TAD could induce a structuring effect in the anchor region that restores the original structural mobility in the ATP and nucleoside binding regions to produce higher kinase activity. However, our data suggests that a protein accumulation method is more likely to be the underlying mechanism for TICKLE's switching.

The protein accumulation mechanism has been seen with protein switches designed in the past using the CH1 as the signal recognition domain [12,16]. These previous studies concerned the development of protein switches using yCD as the output domain and showed effector-enabled toxicity in both bacterial and mammalian systems. Cytosine deaminase, in combination with 5- 5-FC, is prominently used in GDEPT studies and its synergy with HSV-TK and ganciclovir (GCV) is well documented [13,39,40]. The toxicity produced from these yCD switches is not as high as from wild-type yCD. While the efficacy of TICKLE in mammalian systems needs to be tested, an HSV-TK-based protein switch could be used in combination with a yCD-based protein switch to produce a synergistic effect. HSV-TK's broad substrate specificity is key to this synergy; it allows faster metabolism of substrates activated by yCD and may, in fact, result in higher toxicity levels produced directly from 5-FC [41]. This broad specificity may also exist in TICKLE. Beyond implications in cancer therapy, HSV-TK has also been investigated for its potential as a reporter gene in animal models and clinical studies [17,18,42]. The kinase, in combination with 2'-[18F]fluoro-5-ethyl-1-beta-D-arabinofuranosyluracil (18F-FEAU), has been utilized in positron imaging tomography (PET). The difference in activity levels based on HIF1α availability in TICKLE would also make the switch an attractive candidate to be tested for the purpose of medical imaging and may be able to identify hypoxic environments in animal models more efficiently.

Mutational analysis of the linker regions of TICKLE indicated that the linker composition plays an important role in the protein's ability to behave as a switch. As

seen with TICKLE (S243G, T244G, T245S), mutations to the linker abolished almost all activity regardless of the presence of C-TAD. A construct consisting of single point mutation in TICKLE (T245S) at the linker region resulted in reduced activity in the "ON" state and decreased switching efficiency by 2-fold. These results underscore the influence of the linkers in sensitizing TICKLE to an effector molecule. The sensitivity of the linker to mutation suggests that linker optimization might lead to a 2nd generation TICKLE switch that has superior switching properties. A combinatorial linker library combined with our two-tiered selection could be used for such optimization.

In addition, the binding promiscuity of CH1 must also be addressed. The CH1 domain has binding affinity towards other transcription factors besides HIF1-α [16,43]. Other binding partners might also activate TICKLE. We have previously shown that a CH1-yCD protein switches created in our lab can be activated by CITED2 in *E. coli,* and we suspect that activation of this switch in Flp-In 293 human cells in the absence of HIF1-α accumulation might result from binding of some other protein besides HIF1-α [16]. However, while the binding of transcription factors other than HIF1-α may result in the activation of TICKLE, the main binding partners of CH1 are up-regulated in cancerous environments and may still induce increased toxicity towards cancer cells [16,44,45].

We have established a kinase-based proteins switch that is capable of generating increased prodrug toxicity towards *E. coli* cells in the presence of the C-TAD domain of HIF1-α. The selective toxicity based on the presence of hypoxia-induced elements makes TICKLE an appealing candidate for future investigation in

mammalian cancer systems. While the enzymatic switch may not yet have sufficient differences between its “on” and “off” states and may suffer from effector specificity issues, further rounds of directed evolution may solve these issues. With the broad substrate specificity provided by the HSV-TK domain, the enzymatic switch may be capable of activating an array of substrates in a switch-like fashion and could be utilized for a variety of applications.

Table 3.1: HSV-TK inverse PCR primers with corresponding target codons.

Target Codon	Forward Primer	Reverse Primer
HSV-TK 1	atggcttcgtacccctgcca	acgcgcttctacaaggc
HSV-TK 2	gcttcgtacccctgccat	catacgcgcttctacaaggc
HSV-TK 3	tcgtacccctgccatca	agccatacgcgcttctaca
HSV-TK 4	tacccctgccatcaacacg	cgaagccatacgcgctt
HSV-TK 5	ccctgccatcaacacgcgtct	gtacgaagccatacgcgct
HSV-TK 6	tgccatcaacacgcgt	ggggtacgaagccatacgcgctt
HSV-TK 7	catcaacacgcgtctgcgt	gcaggggtacgaagcca
HSV-TK 8	caacacgcgtctgcgtt	atggcaggggtacgaagc
HSV-TK 9	cacgcgtctgcgttcgacca	ttgatggcaggggtacga
HSV-TK 10	gcgtctgcgttcgaccaggct	gtgttgatggcaggggtac
HSV-TK 11	tctgcgttcgaccaggct	cgcgtgttgatggcaggggta
HSV-TK 12	gcgttcgaccaggctgcgcgtt	agacgcgtgttgatggc
HSV-TK 13	ttcgaccaggctgcgcgtt	cgcagacgcgtgttga
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HSV-TK 15	caggctgcgcgttctcgcggccat	gtcgaacgcagacgcgtgt
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HSV-TK 17	gcgcgttctcgcggccata	agcctggtcgaacgca
HSV-TK 18	cgttctcgcggccata	cgcagcctggtcgaacgca
HSV-TK 19	tctcgcggccatagcaa	acgcgcagcctggtcgaa
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HSV-TK 21	ggccatagcaaccgacgt	gcgagaacgcgcagcctggt
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HSV-TK 23	agcaaccgacgtacggcgtt	atggccgcgagaacg
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HSV-TK 25	cgacgtacggcgttgcgccctcgccggcagca	gttgctatggccgcgaga
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HSV-TK 41	cgcctggagcagaaaatg	gacttccgtggcttcttgc
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HSV-TK 269	ccccagggtgccgagccccagagc	cggcacggccgtccccgaa
HSV-TK 270	cagggtgccgagccccagagca	gggcggcacggccgtccccgaa
HSV-TK 271	ggtgccgagccccagagcaa	ctggggcggcacggccgtccccgaa
HSV-TK 272	gccgagccccagagcaacgcgggcccacg a	accctggggcggcacggccgtccccgaa
HSV-TK 273	gagccccagagcaacgcgggcccacga	ggcaccctggggcggcacggccgtccccgaa
HSV-TK 274	ccccagagcaacgcgggcccacga	ctcggcaccctggggcggcacggccgtccccg aa
HSV-TK 275	cagagcaacgcgggcccacga	gggctcggcaccctggggcggcacggccgtcc ccgaa
HSV-TK 276	agcaacgcgggcccacgaccccat	ctggggctcggcaccct
HSV-TK 277	aacgcgggcccacgaccccat	gctctggggctcggcaccct
HSV-TK 278	gcgggcccacgaccccatat	gttgctctggggctcggcaccct
HSV-TK 279	ggcccacgaccccatat	cgcgttgctctggggctcggcaccct
HSV-TK 280	ccacgaccccatatcggggaca	gcccgcgttgctctg
HSV-TK 281	cgaccccatatcggggaca	tgggcccgcgttgctct
HSV-TK 282	ccccatatcggggacacgt	tcgtgggcccgcgttgctc
HSV-TK 283	catatcggggacacgttatttacc	gggtcgtgggcccgcgttgctct
HSV-TK 284	atcggggacacgttatttaccc	atggggtcgtgggcccgcgtt
HSV-TK 285	ggggacacgttatttaccctg	gatatggggtcgtgggcccgcgtt
HSV-TK 286	gacacgttatttaccctgtttcgg	cccgatatggggtcgtgggcccgcgtt
HSV-TK 287	acgttatttaccctgtttcggg	gtccccgatatggggtcgtg
HSV-TK 288	ttatttaccctgtttcgggcc	cgtgtccccgatatggggtcgt
HSV-TK 289	tttaccctgtttcgggcc	taacgtgtccccgatatggg
HSV-TK 290	accctgtttcgggcccccgagt	aaataacgtgtccccgatatgg
HSV-TK 291	ctgtttcgggcccccgagt	ggtaaataacgtgtccccgatatg
HSV-TK 292	tttcgggcccccgagtt	cagggtaaataacgtgtcccc

HSV-TK 293	cgggcccccgagttgctggcccccaa	aaacagggtaaataacgtgtcccc
HSV-TK 294	gcccccgagttgctggcccccaa	ccgaaacagggtaaataacgtgt
HSV-TK 295	cccgagttgctggcccccaa	ggcccgaaacagggtaaa
HSV-TK 296	gagttgctggcccccaa	gggggcccgaaacagggt
HSV-TK 297	ttgctggcccccaacggcgacct	ctcgggggcccgaaa
HSV-TK 298	ctggcccccaacggcgacctgt	caactcgggggcccgaa
HSV-TK 299	gcccccaacggcgacctgta	cagcaactcgggggcccgaa
HSV-TK 300	cccaacggcgacctgta	ggccagcaactcgggggcccgaa
HSV-TK 301	aacggcgacctgtataacgt	gggggccagcaactcgggggcccgaaa
HSV-TK 302	ggcgacctgtataacgtgtttg	gttgggggccagcaact
HSV-TK 303	gacctgtataacgtgtttgcctg	gccgttgggggccagcaact
HSV-TK 329	tacgaccaatcgcccgccggctgccgggacgccctgct	atccaggataaagacgtgcatg
HSV-TK 330	gaccaatcgcccgccggctgccgggacgccctgct	gtaatccaggataaagacgtgcatg
HSV-TK 331	caatcgcccgccggctgccgggacgccctgct	gtcgtaatccaggataaagacgtg
HSV-TK 332	tcgcccgccggctgccgggacgccctgct	ttggtcgtaatccaggataaagacg
HSV-TK 333	cccgccggctgccgggacgccctgctg	cgattggtcgtaatccagga
HSV-TK 334	gccggctgccgggacgccctgctgca	gggcgattggtcgtaatcc
HSV-TK 335	ggctgccgggacgccctgctgcaa	ggcgggcgattggtc
HSV-TK 336	tgccgggacgccctgctgcaa	gccggcgggcgattggt
HSV-TK 337	cgggacgccctgctgcaact	gcagccggcgggcgatt
HSV-TK 338	gacgccctgctgcaact	ccggcagccggcgggcgattgg
HSV-TK 339	gccctgctgcaacttacc	gtcccggcagccggcgggcgatt
HSV-TK 340	ctgctgcaacttacctccg	ggcgtcccggcagccggcgggcgatt
HSV-TK 341	ctgcaacttacctccggga	cagggcgtcccggcagccggcgggcgat
HSV-TK 342	caacttacctccgggatgg	cagcagggcgtcccggcagccggcgggcgat
HSV-TK 343	cttacctccgggatggtcca	ttgcagcagggcgtcccggcagccggcgggcgat
HSV-TK 344	acctccgggatggtccaga	aagttgcagcagggcgt
HSV-TK 345	tccgggatggtccagaccca	ggtaagttgcagcagggcgtcccggcagccggcgggcgat
HSV-TK 346	gggatggtccagacccacgt	ggaggtaagttgcagcagg
HSV-TK 347	atggtccagacccacgt	cccggaggtaagttgcag
HSV-TK 348	gtccagacccacgtcacca	catcccggaggtaagttgc
HSV-TK 349	cagacccacgtcaccacccccggctcca	gaccatcccggaggtaagt
HSV-TK 350	acccacgtcaccacccccggctcca	ctggaccatcccggaggt
HSV-TK 351	cacgtcaccacccccggctcca	ggtctggaccatcccggaggt
HSV-TK 352	gtcaccacccccggctccat	gtgggtctggaccatccc
HSV-TK 353	accacccccggctccata	gacgtgggtctggacca
HSV-TK 354	acccccggctccataccga	ggtgacgtgggtctgga
HSV-TK 355	cccggctccataccgacga	ggtggtgacgtgggtct
HSV-TK 356	ggctccataccgacgatatg	gggggtggtgacgtgggtct
HSV-TK 357	tccataccgacgatatgcga	gccgggggtggtgacgtgggtct

HSV-TK 358	ataccgacgatatgcgacct	ggagccgggggtggtgacgt
HSV-TK 359	ccgacgatatgcgacctg	tatggagccgggggtggt
HSV-TK 360	acgatatgcgacctggcgcgcacgt	cggtatggagccgggggtggt
HSV-TK 361	atatgcgacctggcgcgcacgt	cgtcggtatggagccgggggtggt
HSV-TK 362	tgcgacctggcgcgcacgtt	tatcgtcggtatggagccg
HSV-TK 363	gacctggcgcgcacgttt	gcatatcgtcggtatggagc
HSV-TK 364	ctggcgcgcacgtttgcccgggaga	gtcgcatatcgtcggtatgg
HSV-TK 365	gcgcgcacgtttgcccgggaga	caggtcgcatatcgtcggt
HSV-TK 366	cgcacgtttgcccgggaga	cgccaggtcgcatatcg
HSV-TK 367	acgtttgcccgggaga	gcgcgccaggtcgcatat
HSV-TK 368	tttgcccgggagatgggggaggct	cgtgcgcgccaggtcgca
HSV-TK 369	gcccgggagatgggggaggcta	aaacgtgcgcgccaggt
HSV-TK 370	cgggagatgggggaggcta	ggcaaacgtgcgcgccaggt
HSV-TK 371	gagatgggggaggctaact	ccgggcaaacgtgcgcgccaggt
HSV-TK 372	atgggggaggctaactga	ctcccgggcaaacgtgcgcgccaggt
HSV-TK 373	ggggaggctaactgaaacac	catctcccgggcaaacgt
HSV-TK 374	gaggctaactgaaacacggaag	ccccatctcccgggcaaacgt
HSV-TK 375	gctaactgaaacacggaaggag	ctcccccatctcccgggcaaacgt
HSV-TK 376	aactgaaacacggaaggagaca	agcctcccccatctcccgggcaaa

Table 3.2: HSV-TK/CH1 switch names and corresponding DNA sequences

Switch Name	DNA Sequence
TICKLE 1B12	ATGGCTTCGTACCCCTGCCATCAACACGCGTCTGCGTTCGACCAGGCTGCGCGTTCTCGCGGCC ATAGCAACCGACGTACGGCGTTGCGCCCTCGCCGGCAGCAAGAAGCCACGGAAGTCCGCCTGGA GCAGAAAATGCCCACGCTACTGCGGGTTTATATAGACGGTCCTCACGGGATGGGGAAAACCAC CACCACGCAACTGCTGGTGGCCCTGGGTTCGCGCGACGATATCGTCTACGTACCCGAGCCGATG ACTTACTGGCAGGTGCTGGGGGCTTCCGAGACAATCGCGAACATCTACACCACACAACACCGCC TCGACCAGGGTGAGATATCGGCCGGGGACGCGGCGGTGGTAATGACAAGCGCCCAGATAACAA TGGGCATGCCTTATGCCGTGACCGACGCCGTTCTGGCTCCTCATATCGGGGGGGAGGCTGGGAG CTCAAGTGGTGGAGACCCTGAGAAGAGGAAACTGATCCAGCAGCAGCTGGTGCTGCTGCTGCA CGCCCATAAGTGCCAGAGGAGAGAGCAGGCTAACGGGGAAGTGAGGCAGTGCAATCTGCCACA CTGTAGAACCATGAAGAACGTGCTGAATCACATGACACATTGCCAGTCTGGCAAAAGTTGCCA GGTGGCCCATTGTGCTAGCTCCCGGCAGATCATTAGCCACTGGAAGAACTGTACCCGCCATGAC TGCCCCGTGTGTCTGCCTCTGAAGAATGCTTCAACCACCCATGCCCCGCCCCCGGCCCTCACCCT CATCTTCGACCGCCATCCCATCGCCGCCCTCCTGTGCTACCCGGCCGCGCGGTACCTTATGGGC AGCATGACCCCCCAGGCCGTGCTGGCGTTCGTGGCCCTCATCCCGCCGACCTTGCCCGGCACCA ACATCGTGCTTGGGGCCCTTCCGGAGGACAGACACATCGACCGCCTGGCCAAACGCCAGCGCCC CGGCGAGCGGCTGGACCTGGCTATGCTGGCTGCGATTCGCCGCGTTTACGGGCTACTTGCCAAT ACGGTGCGGTATCTGCAGTGCGGCGGGTCGTGGCGGGAGGACTGGGGACAGCTTTCGGGGACG GCCGTGCCGCCCCAGGGTGCCGAGCCCCAGAGCAACGCGGGCCCACGACCCCATATCGGGGACA CGTTATTTACCCTGTTTCGGGCCCCCGAGTTGCTGGCCCCCAACGGCGACCTGTATAACGTGTT TGCCTGGGCCTTGGACGTCTTGGCCAAACGCCTCCGTTCCATGCACGTCTTTATCCTGGATTAC GACCAATCGCCCGCCGGCTGCCGGGACGCCCTGCTGCAACTTACCTCCGGGATGGTCCAGACCC ACGTCACCACCCCCGGCTCCATACCGACGATATGCGACCTGGCGCGCACGTTTGCCCGGGAGAT GGGGGAGGCTAACTGA
TICKLE 2A4	ATGGCTTCGTACCCCTGCCATCAACACGCGTCTGCGTTCGACCAGGCTGCGCGTTCTCGCGGCC ATAGCAACCGACGTACGGCGTTGCGCCCTCGCCGGCAGCAAGAAGCCACGGAAGTCCGCCTGGA GCAGAAAATGCCCACGCTACTGCGGGTTTATATAGACGGTCCTCACGGGATGGGGAAAACCAC CACCACGCAACTGCTGGTGGCCCTGGGTTCGCGCGACGATATCGTCTACGTACCCGAGCCGATG ACTTACTGGCAGGTGCTGGGGGCTTCCGAGACAATCGCGAACATCTACACCACACAACACCGCC TCGACCAGGGTGAGATATCGGCCGGGGACGCGGCGGTGGTAATGACAAGCGCCCAGATAACAA TGGGCATGCCTTATGCCGTGACCGACGCCGTTCTGGCTCCTCATATCGGGGGGGAGGCTGGGAG CTCAAGTGGTGGAGACCCTGAGAAGAGGAAACTGATCCAGCAGCAGCTGGTGCTGCTGCTGCA CGCCCATAAGTGCCAGAGGAGAGAGCAGGCTAACGGGGAAGTGAGGCAGTGCAATCTGCCACA CTGTAGAACCATGAAGAACGTGCTGAATCACATGACACATTGCCAGTCTGGCAAAAGTTGCCA GGTGGCCCATTGTGCTAGCTCCCGGCAGATCATTAGCCACTGGAAGAACTGTACCCGCCATGAC TGCCCCGTGTGTCTGCCTCTGAAGAATGCTTCCACCACTCATGCCCCGCCCCCGGCCCTCACCCT CATCTTCGACCGCCATCCCATCGCCGCCCTCCTGTGCTACCCGGCCGCGCGGTACCTTATGGGC AGCATGACCCCCCAGGCCGTGCTGGCGTTCGTGGCCCTCATCCCGCCGACCTTGCCCGGCACAA ACATCGTGCTTGGGGCCCTTCCGGAGGACAGACACATCGACCGCCTGGCCAAACGCCAGCGCCC CGGCGAGCGGCTGGACCTGGCTATGCTGGCTGCGATTCGCCGCGTTTACGGGCTACTTGCCAAT ACGGTGCGGTATCTGCAGTGCGGCGGGTCGTGGCGGGAGGACTGGGGACAGCTTTCGGGGACG GCCGTGCCGCCCCAGGGTGCCGAGCCCCAGAGCAACGCGGGCCCACGACCCCATATCGGGGACA CGTTATTTACCCTGTTTCGGGCCCCCGAGTTGCTGGCCCCCAACGGCGACCTGTATAACGTGTT TGCCTGGGCCTTGGACGTCTTGGCCAAACGCCTCCGTTCCATGCACGTCTTTATCCTGGATTAC GACCAATCGCCCGCCGGCTGCCGGGACGCCCTGCTGCAACTTACCTCCGGGATGGTCCAGACCC ACGTCACCACCCCCGGCTCCATACCGACGATATGCGACCTGGCGCGCACGTTTGCCCGGGAGAT GGGGGAGGCTAACTGA

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Chapter 4: Methods of metabolic manipulation in *E. coli* for HSV-TK activity selection.

Introduction

The previous three chapters have provided a thorough coverage of the potential applications of HSV-TK. The ability of HSV-TK to activate prodrugs in cancer therapy and imaging substrates for PET imaging provides the incentive to engineer the enzyme towards specific goals, such as higher activity towards specific prodrugs or lower affinity towards thymidine. The capacity to use directed evolution to identify variants with desired activity levels is greatly facilitated by a reliable positive selection or screening. A robust positive selection permits a more efficient process for variant identification in directed evolution when compared to screens. Screens can be a tedious process since variants need to be tested individually instead of en-masse. This problem can be partially alleviated by using robots to aid in the screening, but accessibility to robotic equipment is not always available. The typical screen for HSV-TK activity utilizes testing variants in the presence of prodrugs and observing the level of activity through *in vivo* cell viability assays or *in vitro* coupled enzyme assays (1). The *in vitro* assay can take 3-4 hours per variant to complete but also requires additional time to purify the protein and the *in vivo* assay may take up to 24 hours. Testing thousands of variants through these assays drastically lengthens the total time to identifying successful hits in the screen. In contrast, a reliable positive selection can test millions of variants within 24 hours.

Using bacterial systems for directed evolution is a dramatically faster method for engineering HSV-TK enzymes when compared to mammalian systems. Bacteria grow faster and require less maintenance when compared to mammalian cells. The biggest advantage of using bacterial systems, however, is that large DNA libraries of HSV-TK variants can be easily incorporated into bacterial cells while mammalian cell transfection methods are drastically less efficient. There are, however, disadvantages to using bacterial cells. Engineering HSV-TK in bacteria for the purpose of utilization in mammalian cells is an indirect method and can be ineffective if the bacteria does not possess the required cellular conditions for certain enzyme-substrate interactions that are found in mammalian cells. However, it is possible to overcome this drawback by manipulating the bacterial systems to produce these cellular conditions by co-expressing specific mammalian genes with the HSV-TK variants.

Selecting for the thymidine kinase activity of HSV-TK in bacterial systems requires inhibiting endogenous production of thymidine monophoshate (dTMP). *E. coli* has two endogenous pathways for dTMP production: one utilizes a thymidylate synthase enzyme (thyA) to methylate deoxyuridine monophosphate (dUMP) to produce dTMP and the second uses an endogenous thymidine kinase (Tdk) to phosphorylate dT in to dTMP as a salvage mechanism (Fig. 4.1) (3). Disrupting both of these pathways renders the cell non-viable through a process known as "thymineless death". Thymineless death is a well-characterized event where deoxythymidine triphosphate (dTTP) deficiency results in inhibition of cell

replication and DNA synthesis (4). Without enough dTMP to be converted into dTTP, the bacteria experience this state. Up until recently, the best positive selection for HSV-TK activity utilized *E. coli* KY895, a –*tdk* strain, treated with 5-fluorodeoxyuridine (5-FdU) (5). 5-FdU directly inhibits the thyA enzyme. With *tdk* knocked out and thyA inhibited, the burden of producing enough dTMP for the cell to survive falls on an active HSV-TK variant supplied in trans on a plasmid (2). This selection scheme is feasible, as shown in multiple studies, but suffers from a major drawback. The 5-FdU used to inhibit thyA is also capable of being phosphorylated by HSV-TK into 5-FdUMP (6). 5-FdUMP can be further processed by cellular kinases to its triphosphate form and cause cytotoxicity by getting incorporated into DNA during DNA synthesis (6). The metabolization of 5-FdU by HSV-TK places a restriction on the highest amount of HSV-TK activity that can be selected for. Variants that possess too high an activity would generate sufficient 5-FdUMP to kill the host cell and so, the selection only generates variants that possess lower than optimal activity levels. This issue is compounded by 5-FdU's mutagenic effects, which could induce a higher false positive rate in the selections. Due the restrictions presented by 5-FdU being both a thyA inhibitor and an HSV-TK activated metabolite and also being mutagenic, a new selection scheme that does not hamper the identification of high activity HSV-TK variants is desirable. Chapter two covered an alternative approach that relied on exporting HSV-TK variants into the periplasm and selecting for kinase activity through resistance to AZT that is generated from HSV-TK converting AZT to AZT-MP that is not able to pass the inner membrane of *E. coli* due to its negative charge. Engineering HSV-TK for certain purposes, as a

protein switch that recognizes cytoplasmic signals for example, makes exporting HSV-TK containing fusions into the periplasmic space a non-option. In this scenario, inhibiting endogenous dTMP production in the cell may be the best way to identify HSV-TK variants with high kinase activity. Here, we report on three different potential positive selection schemes that we tested which utilized the *E. coli* KY895 (-*tdk*) strain and inhibited the thyA enzyme in different ways.

The first method involves genetic engineering of *E. coli* KY895 to produce a –*tdk* and –*thyA* strain that has a conditionally expressible copy of *thyA* on a plasmid. These conditions allow the strain to lose its viability when *thyA* expression is repressed and the burden of producing dTMP falls on the plasmid-borne HSVK-TK variants (Fig. 4.1). The second scheme involves inhibiting endogenous thyA in *E. coli* KY895 through raltitrexed, a potent inhibitor of the enzyme. Raltitrexed (TDX) is an antifolate drug that binds to the methylene-tetrahydrofolate (m-THF) binding site of thyA (8). The thyA enzyme uses m-THF as a cofactor to be oxidized during the methylation of deoxyuridine monophosphate (dUMP). Raltitrexed's competitive inhibition of its binding site stops the redox capability of thyA and the production of dTMP. The third scheme that was tested used the antibiotic trimethoprim in *E. coli* KY895. Trimethoprim (TMT) prevents tetrahydrofolate production by inhibiting dihydrofolate reductase (DHFR), an enzyme that catalyzes the reduction of dihydrofolates into tetrahydrofolates (Fig. 4.2) (7). The resulting tetrahydrofolate deficiency restricts thyA from methylating dU and creating dTMP. All three of these

schemes were tested in their ability to shutdown endogenous dTMP production and have it generated through HSV-TK supplied in trans.

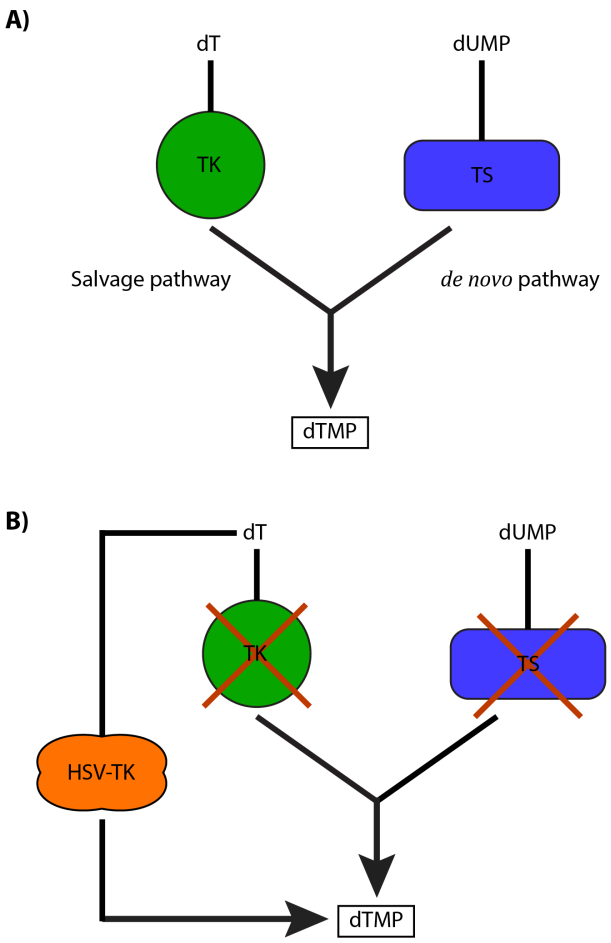


Figure 4.1: Endogenous dTMP production in *E. coli.*

A) *E. coli* utilizes thymidylate synthase (TS) to methylate deoxyuridine monophosphate (dUMP) and produce deoxythymidine monophosphate (dTMP) in the *de novo* pathway. It can also use an endogenous thymidine kinase (TK) to phosphorylate exogenous thymidine (dT) and create dTMP in the salvage pathway. B) Inhibiting both the salvage and *de novo* pathways results in dTMP deficiency and eventually leads to thymineless death. An active HSV-TK can substitute for the inhibited TK and provide the necessary dTMP to alleviate this condition in the cell.

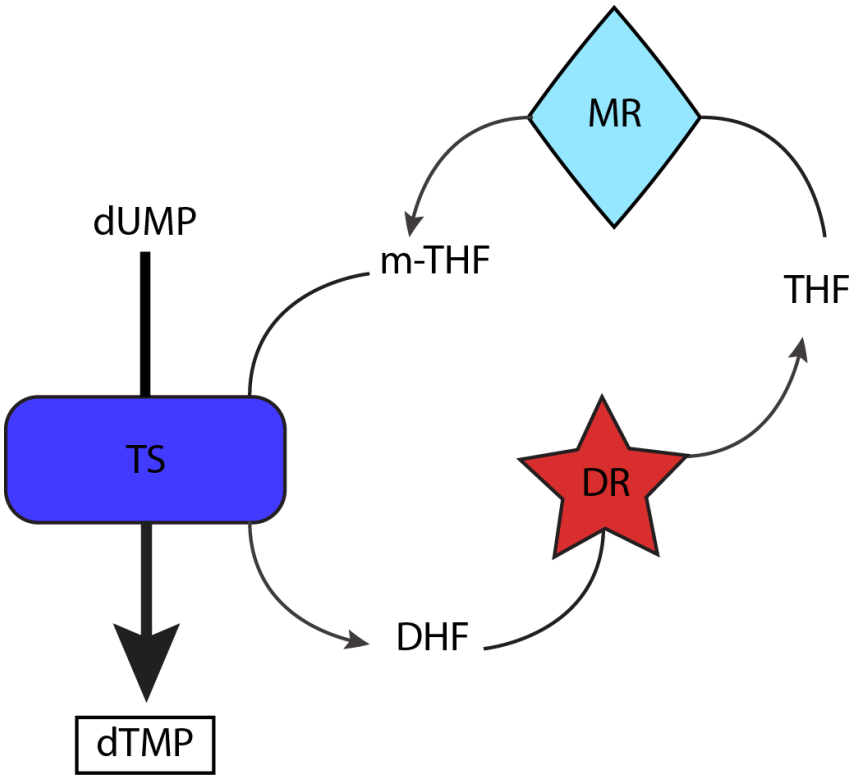


Figure 4.2: Utilization of folate in dTMP biosynthesis.

In order for TS to methylate dUMP, dihydrofolate (DHF) is first reduced by dihydrofolate reductase (DR) to create tetrahydrofolate. The tetrahydrofolate has a methylene moiety attached to it by methylene-tetrahydrofolate reductase (MR) to create the methylene-tetrahydrofolate (m-THF). The m-THF compound is utilized by TS as a cofactor donating the methyl group to be attached to dUMP. During the process of methylating dUMP, the m-THF is converted back to DHF and the cycle continues.

Materials & Methods

Unless otherwise specified, all molecular biology protocols were performed using New England Biolab's (NEB) High-Fidelity Phusion Master Mix for PCR, Invitrogen's Gel Purification Kit for gel extraction and Zymo's DNA Clean & Concentrator kit for DNA purification (5 µg loading capacity). Qiagen's QIAprep Spin Miniprep Kit was used to isolate plasmid DNA from cell culture. 3'-Azido-3'-Deoxythymidine (Azidothymidine, AZT) was purchased from Sigma-Aldrich. 5'-

Triphosphate -3'-Azido-3'-Deoxythymidine (AZT-TP) was obtained through US Biological Life Sciences. Ganciclovir (GCV) was purchased from Sigma-Aldrich. All nucleotide oligomers were ordered from Integrated DNA Technologies. Ligations were performed using New England Biolabs' T4 DNA Ligase Buffer (10x) and T4 DNA Ligase (400,000 units/mL).

Development of E. coli NS01 strain

The vector pRHAM.thyA was used to complement *E. coli* NS01 (-*tdk, -thyA*) strain that was developed. The pRHAM plasmid contains a chloramphenicol resistance marker (CmR), a rhamnose inducible promoter, a low copy p15 origin and has been characterized before (17). The *thyA* gene of *E. coli* was amplified from *E. coli* KY895 genome in a PCR using primers that bind to the start and stop codons of the gene. The pRHAM vector was linearized using inverse PCR that opened the plasmid 20 base pairs (bp) downstream of the rhamnose promoter and the *thyA* gene was blunt-ligated into the linearized plasmid as per NEB's T4 DNA Ligase protocol. After DNA purification of the ligation reaction, the pRHAM.thyA was transformed into *E. coli* KY895. Colonies were sequence verified the next morning.

The vector pKD46, which houses the Lambda Red Recombinase system for genetic engineering was transformed into *E. coli* KY895 with pRHAM.thyA. The pKD46 vector is temperature sensitive and does not replicate above 30°C so the transformants were grown at that temperature. The entire Lambda Red Recombinase system for gene deletions has been characterized and elaborated on in previous studies (9). A linear kanamycin resistance gene (*KanR*) with 50 bp flanks possessing homology to the upstream and downstream DNA of endogenous *thyA*

was then transformed into the same bacteria. The *KanR* gene was amplified from *E. coli* JW1226, a strain purchased from Yale's *Coli* Stock Center, with primers that possessed the corresponding 50 bp flanks. The transformants were grown in 0.2% arabinose (to induce the Recombinase expression) and 0.2% rhamnose (to induce expression of the complementary *thyA* on pRHAM.thyA) at 30°C on 50 μg/mL Kan. Colonies that grew in these conditions needed to possess a *KanR* gene insertion in the genome.

The colonies were screened on "Permissive" and "Non-permissive" plates to confirm the deletion of endogenous *thyA* and proper complementation of the plasmid copy of *thyA.* The permissive plates had 2% rhamnose and 10 μg/mL dT with 50 μg/mL Kan and 34 μg/mL Cm on LB agar. The non-permissive plates had 2% glucose and 10 μg/mL dT with 50 μg/mL Kan and 34 μg/mL Cm on LB agar. These were incubated at 37°C to remove the pKD46 plasmid from the system. Once the genotype of *E. coli* NS01 strain (-*tdk, -thyA*) was confirmed, pMCC was transformed into it in order to test the positive selection of thymidine kinase activity. The pMCC vector holds *hsv-tk* and was elaborated on in Chapter 2 and 3. Plates with the permissive and non-permissive conditions described above and 50 μg/mL ampicillin (Amp) were plated with *E. coli* NS01 containing pMCC to compare survival of colonies when endogenous dTMP production is present and absent in the presence of HSV-TK.

Raltitrexed Selection

E. coli KY895 cells with pMCC or pMDC (a variant with an active site deletion in *hsvtk*, described in Ch. 2 and Ch. 3) were plated on solid tryptone media (TM) containing 50 μg/mL Amp and 0, 5.7, 11.4, 22.8 μg/mL of TDX. The plates were incubated at 37°C for 18 hours. The growth on each plate was then observed to compare the difference between an active HSV-TK (pMCC) and an inactive HSV-TK (pMDC). Liquid media experiments using TM with 50 μg/mL Amp and 0, 5.7, 11.4, 22.8 μg/mL of raltitrexed were conducted. One thousands CFUs of *E. coli* KY895 housing pMCC or pMDC were inoculated in liquid cultures and incubated overnight at 37°C for 18 hours. The optical densities (OD) was measured for each culture the next morning and compared to the control (0 μg/mL TDX).

Trimethoprim Selection

E. coli KY895 cells with pMCC or pMDC were plated on solid Luria Broth media (LB) containing 50 μg/mL Amp and 0, 1, 2.5, 10 μg/mL of trimethoprim (TMT). The plates were incubated at 37°C for 18 hours. The growth on each plate was then observed to compare the difference between an active HSV-TK (pMCC) and an inactive HSV-TK (pMDC).

Results & Discussion

Genetic inhibition selection scheme

We developed *E. coli* NS01 as a –*thyA* and –*tdk* strain using the Lambda Red Recombinase genetic engineering system. The endogenous *thyA* from *E. coli* KY895

and replaced with *KanR.* A complementary copy of *thyA* was provided on a plasmid and was able to salvage the viability of *E. coli* NS01 in the presence of rhamnose (Fig. 4.3). In repressive conditions featuring glucose and no rhamnose, the strain was not able to survive. Screens in LB agar and Amp confirmed that the original pKD46 plasmid housing the Lambda Red Recombinase was removed from the cells by growing them at 37°C and could not skew any results. While the complementary *thyA* proved to successfully keep the cells viable, HSV-TK was not able to provide the necessary levels of dTMP in non-permissive conditions to sustain viability (Fig. 4.4). The likely reason for this is that the strain of plasmid maintenance and replication during dTMP deficiency is too high for cell to overcome with HSV-TK activity. The 6854 bp pMCC vector has a ColE origin, which makes approximately 300 copies inside the cell (10). The net DNA length of all pMCC vector copies equals more than 2 megabases (Mb); considering that the *E. coli* genome is approximately 5 Mb, this would indicate a 40% increase in dTMP required for cell replication if sequence composition distribution were considered identical between the genome and the plasmid. It could be very likely that the significant rise in required dTMP cannot be supplied by HSV-TK alone. Further optimization of plasmid type and promoter type for HSV-TK expression may allow for high enough concentrations of HSV-TK from a low copy number plasmid to rescue the dTMP deficient cells.

Permissive Non-permissive

Figure 4.3:

Selected colonies of *E. coli* NS01 (previously called NS1α) streaked on permissive (+rhamnose) and non-permissive (+glucose) plates. All variants displayed conditional viability, variant #3 was used for further investigation into HSV-TK rescue of cell viability.

Raltitrexed Selection Scheme

Using raltitrexed (TDX) to inhibit thyA of *E. coli* was not successful. In the absence of HSV-TK activity, raltitrexed was expected to make the strain non-viable and there should have been no growth. However, our studies revealed that colonies were able to grow on plates containing cells with HSV-TK and cells with an inactive HSV-TK, even at the highest raltitrexed concentration (22.8 μg/mL) (Fig. 4.5). The liquid culture assays also showed no drastic difference in OD between the control and cultures exposed TDX. Considering that the IC50 of TDX is 9 nM in LC1210

leukemia cells, which equates to 4.1 ng/mL, the lack of toxicity produced is surprising (11). While the thymidylate synthase of leukemia cells and *E. coli* are different in structure, the bacterial thymidylate synthase has been implicated in interacting with TDX (12). Due to this, the lack of toxicity is not asserted to be due to a lack of thyA-TDX interaction. Instead, the deficiency in toxicity is suspected to be due to the nature of folate usage in bacteria. Humans cannot synthesize folates and need to scavenge the tetrahydrofolate precursor, dihydrofolate (vitamin B_9), from the environment (13). This is why vitamin B_9 is considered an essential vitamin. In contrast, bacteria can produce their own dihydrofolate through the enzyme dihydropteroate synthase (14). With the capacity to produce dihydrofolate internally, the *E. coli* do not have a known designated protein for folate uptake (15). Due to this, perhaps raltitrexed cannot be internalized since it is a folate analog. However, certain bacteria do not synthesize their own folates and require exogenous folates for survival. For example, *Lactobacillus casei* (*L. casei*) used *folT,* a folate and thiamine transport protein to internalize folates (16). In future studies, this *folT* gene could be cloned into *E. coli* in order to use raltitrexed in a potential positive selection scheme for HSV-TK.

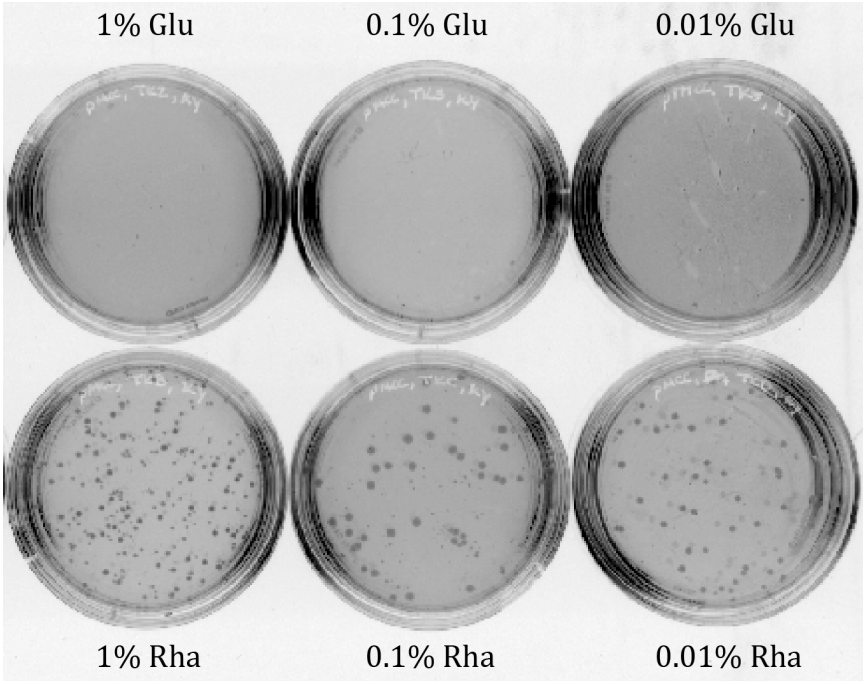


Figure 4.4:

E. coli KY895 with pMCC was on various permissive and non-permissive conditions to observe HSV-TK ability to rescue cell from dTMP deficiency. Rhamnose (Rha) levels of 1%, 0.1%, 0.01% were used for permissive conditions. Glucose (Glu) levels of 1%, 0.1%, 0.01% were used for non-permissive conditions.

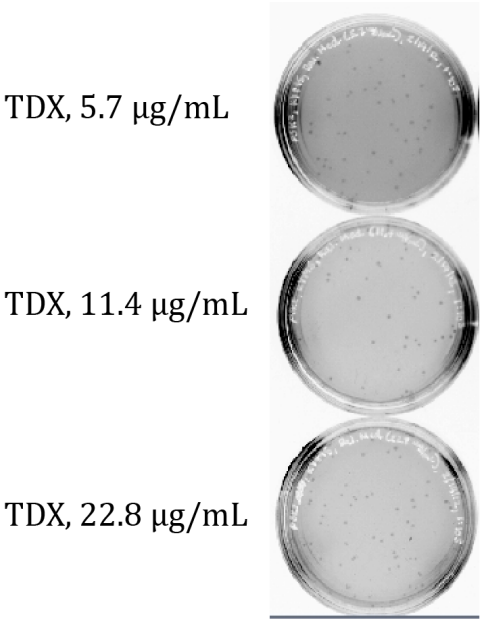


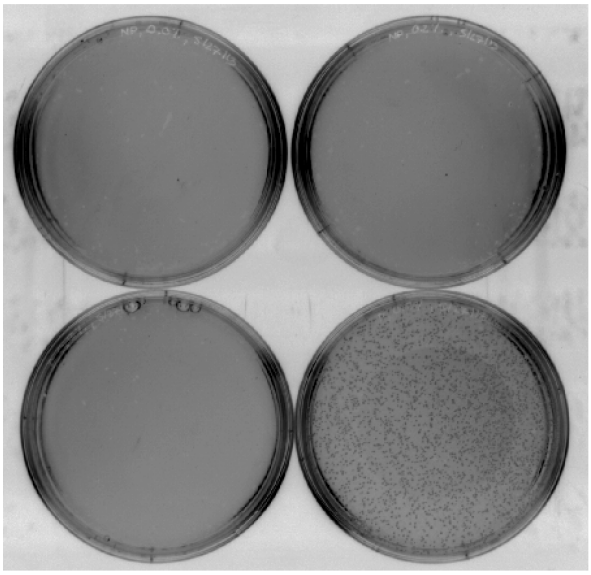
Figure 4.5:

Raltitrexed (TDX) at concentrations of 5.7, 11.4, 22.8 μg/mL on tryptone media plates. Approximately 100 cfus of *E. coli* KY895 plated on each plate. Plate with TDX concentration of 0 μg/mL not shown.

Trimethoprim Selection Scheme

In the TMT selection for HSV-TK activity, the underlying concept was to hamper folate production in order to disable the activity of thymidylate synthase. TMT directly inhibits DHFR. Our results showed no bacterial growth for cells containing HSV-TK and an inactive HSV-TK, even at the lowest concentrations of TMT (1 μg/mL) (Fig. 4.6). The outcome indicates that TMT is extremely toxic to *E. coli.* Tetrahydrofolate is used in many enzymes as a cofactor that enables redox reactions. Restricting tetrahydrofolate production adversely affects more enzymes than just thyA, which may be the reason why HSV-TK cannot overcome the resulting dTMP deficiency. We suspect this strategy to create a positive selection failed because it does not directly and solely influence thyA activity. Lower levels of TMT may be able to properly inhibit thyA without rendering the cells non-viable, but the lack of thyA-specific inhibition would require a high degree of optimization in order to keep the cell inducing stress responses that could comprise the integrity of the positive selection.

1 µg/mL TMP 2.5 µg/mL TMP



10 µg/mL TMP 0 µg/mL TMP

Figure 4.6:

1000 cfus of *E. coli* KY895 with pMCC were plated on LB agar containing 0.2% glucose and different levels of TMP.

Of the three approaches that have been described in this chapter, the raltitrexed-based HSV-TK activity selection scheme and the *E. coli* NS01 selection scheme are the most promising. Both schemes display specific issues that might be overcome through proper optimization. *E. coli* NS01 requires a more thorough investigation of optimal conditions that express adequate HSV-TK to produce sufficient dTMP while also not stressing the cell too greatly. Changing the plasmid's origin to reduce copy number while simultaneously switching to a stronger promoter may be crucial for this selection to work efficiently. The TDX selection scheme necessitates providing a way for the *E. coli* to internalize folates, an issue that could potentially be resolved by simply cloning in a folate transport gene. The TMT selection scheme, however, shows a major defect in design. The toxicity of the TMT antibiotic presumably affects more than just the thyA enzyme. The non-specific inhibition of thyA makes optimizing all conditions affecting the selection

impractical. Amongst the three approaches that have been described, further work into the raltitrexed-based and the *E. coli* NS01 approaches may yield highly efficient mechanisms to select for HSV-TK activity that do not significantly perturb cellular function.

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Chapter 5: Concluding Remarks & Future Directions

The information presented in this body of work signifies the advances that have been made in engineering of the HSV-TK enzyme, methods of selecting for kinase activity in bacterial systems and providing new insights into protein engineering methodology in directed evolution. The progress reported in the earlier chapters extends on nearly three decades of work in engineering HSV-TK for the purpose of cancer therapy and establishes a strengthened platform for future investigations.

Periplasmic Kinase Selection

One development to aid the engineering of nucleoside kinases by directed evolution was a new method for generating resistance in bacteria towards toxic nucleoside analogs. The method showed that by exporting a nucleoside kinase into the periplasm of *E. coli,* an exogenously added nucleoside analog would be phosphorylated in the periplasm, thereby preventing it from entering the cytoplasm of the bacterium. The enzyme responsible for initiating the toxicity of the nucleoside analog in the cytoplasm becomes responsible for generating the resistance towards the nucleoside analog in the periplasm.

The study demonstrated that significant resistance could be generated towards AZT through periplasmic phosphorylation. Ideally, resistance of a similar magnitude might be produced towards other nucleoside analogs. The resistance generated, however, would be greatly influenced by enzymatic activity of the kinase and speed of internalization of the compound. The competition between nucleoside

analog intake by the cell versus the rate of catalysis of the enzyme responsible for phosphorylating the compound is key to determining the level of resistance. Conceptually, this method can be utilized for any nucleoside kinase that can be active in the periplasm and capable of phosphorylating a nucleoside analog that is normally toxic to *E. coli*. Other therapeutically relevant enzymes, such as cytidine kinase, may be good candidates for this selection scheme.

In its most direct application, the method presumably can be used to select for higher activity variants of these nucleoside kinases. However, there are other potential applications as well. The system could be utilized to select for enzymatic kinase switches, like TICKLE. The requirements to use this selection in switch identification are almost the same as for selecting higher activity enzyme variants; the enzyme needs to be able to possess activity in the periplasmic space but the effector signal responsible for influencing enzymatic activity must also be stable in the periplasm. This is to ensure that the signal can properly interact with the switch variants during the selection. Along with enzymatic kinase switches, this method may also be of use for identifying higher active variants of nucleoside phosphorylases. Certain nucleoside phosphorylases possess therapeutic relevancy, such as thymidine phosphorylase, which can activate the prodrug capecitabine (1). In theory, this selection could also be applicable to such nucleoside phosphorylases, as long as they are stable in the periplasm and capable desphosphorylating essential nucleotides. The method of positive selection process would be inverted for nucleoside phosphorylases. The periplasmic nucleoside phosphorylase would remove phosphates from essential nucleotides to allow them to be transported in

the cytoplasm in nutrient deficient conditions. This would rescue the cell from nucleoside starvation. It should be noted that selecting for phosphorylases may result in a higher false positive rate due to cellular stress responses instigated by the nutrient-deficient environment. Optimization of media conditions and nutrient levels would be needed to keep the cells from generating stress responses capable of significantly skewing the selection.

While this new selection may have broad prospects for protein engineering of kinases and phosphorylases, practical implementation is still lacking. The selection has been optimized around the HSV-TK enzyme and should be applicable to the discovery of high activity HSV-TK variants. A direct subsequent step to this study should be to mutagenize HSV-TK and subject the library to this selection. This would provide authentic scrutiny for the selection, the results of which could be used to further optimization of the method.

Development of TICKLE

The two-tiered selections conducted to identify an enzymatic switch from HSV-TK resulted in the discovery of TICKLE. The switch, in the presence of the C-TAD of HIF1-α, seemed to generate greater toxicity in cells subjected to AZT. The 4-fold increase in toxicity generated from AZT in the presence of the cancer marker denotes the successful coupling of the magnitude of AZT toxicity to presence or absence of the C-TAD. Several yCD-based switches have been made previously in our lab and the development of TICKLE demonstrates that more switches can be generated using CH1 as the signal recognition domain and the HIF1-α C-TAD as the signal. Considering that every switch that has been created by using the CH1/HIF1-

α interaction has relied on a restabilization effect to function, it can be assumed that any future switch would likely also possess this characteristic.

For the purpose of being implemented in cancer therapy, the 4-fold increase in activity observed in TICKLE is a mild difference. The goal of utilizing an enzymatic switch in GDEPT is to abolish off-target activity inside normal cells while preserving high activity in cancer cells. Although it has not been tested in a mammalian system yet, TICKLE, in its current form, would benefit from greater differences in activity between its "On" and "Off" state. Further engineering of the HSV-TK domain of TICKLE would be required in order to increase this difference. Our newly developed periplasmic selection strategy may allow selection for higher levels of kinase activity on AZT that could not be possible in the original positive selection.

A subsequent, logical step in this study would be to investigate TICKLE in a mammalian system. HAPS59, a best-in-class yCD protein switch created in our lab, exhibited a 8-fold switching effect in *E. coli* GIA39 cells (unpublished data). When the switch was tested in mammalian systems, it was able to generate a 7-fold activity difference between normoxic and hypoxic states (2). HSV-TK-based switches such as TICKLE may also maintain the magnitude of their switching effect in cancer cells. Ultimately, mammalian cell line studies will reveal the true potential of TICKLE and any future HSV-TK switches.

The greatest challenge to developing therapeutically-relevant protein switches using CH1 is overcoming the non-specific activation that may occur from domain's binding promiscuity. It has been suggested that the various binding partners of CH1 can activate an enzymatic switch, leading to non-specific toxicity

(3). The most direct way to address this drawback is to engineer the CH1 domain to bind specifically to the C-TAD of HIF1-α and then introduce it back into the output domain. This is a tedious process with uncertain prospects. It is encouraging that the C-TAD of HIF1-α and C-TAD of CITED2 bind to CH1 at different interfaces because mutagenesis could abolish binding selectively to CITED2. However, the CH1 domain is small and there is overlap between the binding interfaces of all of CH1's binding partners. Mutagenesis of CH1 to increase binding specificity towards HIF1-α may also increase the binding affinity of some proteins while decreasing the binding capacity towards others.

Although with proper engineering and testing it may be possible to generate a CH1 variant that binds only to HIF1-α, the difficulty and time-investment required in doing so makes alternative strategies perhaps more appealing. It may be easier to select another protein as a signal recognition domain and use it to develop an enzymatic switch. To pursue this option, a suitable signal recognition domain with the fitting properties attractive must be found. First and foremost, the signal recognition domain must contrast itself from the CH1 domain by only binding to one specific cancer marker. Second, the cancer marker that binds to this protein must be unavailable in regular cells or highly upregulated in cancer cells; this characteristic is to draw a distinct difference between signal availability for the "On" and "Off" states of the enzymatic switch. A third factor to consider is the binding of the signal recognition domain to the cancer marker itself. Preferably, the binding should instigate a large conformational shift in the signal recognition protein or provide a sizeable improvement in stability and structuring. The significance of this factor

relates to the mechanism by which the signal recognition domain influences enzymatic activity in the switch. A considerable change in stability or conformation has a greater chance at impacting the enzymatic domain's activity in a protein switch.

To the best of our knowledge, the creation of TICKLE represents the development of the first engineered nucleoside kinase protein switch. Given HSV-TK's broad applicability, it is of interest as a potential cancer therapeutic and medical imaging agent, although additional protein engineering is needed to increase activity levels between states and generate greater specificity towards prodrugs over dT.

Cytoplasmic Kinase Selection

Our investigations in new cytoplasmic kinase selections for HSV-TK provided some insights into the metabolic manipulation of *E. coli* and its ramifications. The toxicity generated through chemical and genetic inhibition of endogenous dTMP production proved to be difficult to overcome using the activity of HSV-TK. The specific reasons behind this differ between individual selection methods and were elaborated on in Chapter 4.

Conceptually, a successful HSV-TK selection in bacteria involves inhibition of endogenous dTMP production in the *de novo* pathway and the salvage pathway. The three different methods we utilized to achieve targeted inhibition of *de novo* dTMP synthesis in a strain of *E. coli* that was deficient of an endogenous thymidine kinase. Attempts at chemical inhibition of thyA revealed the complexities of using small molecules to disrupt metabolic processes. Metabolically mature antifolates, such as

raltitrexed, which are analogous to folate co-factors, encounter problems crossing into the cytoplasm when they are used for bacterial systems. Simpler antifolates, such as trimethoprim, are analogous to folate precursors, and can be internalized by bacteria. The precursor nature of these antifolates is capable of inhibiting function of enzymes that are dependent on the downstream folate products as co-factors. The non-specific enzymatic inhibition from these simpler antifolates makes them an unfeasible option for a positive selection. The advantage of genetic manipulation over chemical manipulation of bacterial metabolic processes is that it can provide greater target-specific effects.

A genetic deletion of *thyA* proved to be the most successful way of abolishing endogenous dTMP production that also reserved the possibility of rescue through HSV-TK. Studies in Chapter 4 revealed that HSV-TK rescue in solid media was unattainable. These results were in contrast to ones found in the studies undertaken in Chapter 3. Those experiments involved liquid media and showed that HSV-TK is capable of robust rescue of growth of cells with a dTMP deficiency. TICKLE in its "On" state displayed lower, but notable levels of rescue from dT deficiency compared to HSV-TK. The disparity in results between solid and liquid media may be related to the availability dT. The static nature of solid media bacterial growth can lead a depletion of nutrients in the local environment whereas bacteria in liquid culture can scavenge nutrients from the entire culture. This presents a unique situation where a possible positive selection of HSV-TK activity may be possible in liquid media instead of solid media. From a practical standpoint, a properly optimized liquid culture selection can be even more efficient than a solid media

selection. Competition for dT in liquid media would lead to bacterial cells carrying higher activity variants to grow and multiply faster; at the end of the growth period, the higher activity variants would make up a larger portion of the cell culture and be easier to identify upon screening.

Further work is necessary to develop a robustly functioning cytoplasmic kinase selection that can address the limitations of currently available methods. Our investigations highlighted the challenges present in developing such selections and some selection designs that display potential in addressing these challenges.

Final Remarks

This work provides a compilation of developments in engineering HSV-TK. With the primary aim of improving the HSV-TK enzyme's suitability in GDEPT, we transformed the enzyme into TICKLE, a protein switch that may generate HIF1-α-dependent toxicity in cancer cells. In tackling the challenges of engineering HSV-TK in bacteria, our attempts at exploring alternate positive selection schemes highlighted the difficulty of selecting for kinase activity in the cytoplasm. This realization was the inspiration for the periplasmic selection of HSV-TK that we designed, a selection that could be generalized to other nucleoside kinases. Ultimately, our studies offer a new way to engineer the promiscuous HSV-TK enzyme for an array of different purposes and, in the development of TICKLE, establish a baseline HSV-TK enzymatic switch that can be further improved in future GDEPT-related cancer cell studies.

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Curriculum Vitae

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