Developing an Enhanced Screening System for Deoxyguanosine Kinase Mutants with Altered Substrate Specificity

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Spring 2011
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Honors Thesis
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PASS WITH DISTINCTION
Suicide gene therapy is a promising novel approach to cancer treatment that involves the delivery of a suicide gene to cancer cells. Once the suicide gene expresses the "suicide enzyme", a relatively nontoxic prodrug is administered to the patient. The suicide enzyme is able to convert the prodrug to a toxic form, thereby causing death in cancer cells that express the suicide gene. An important part of this process, called the bystander effect, extends the ability of the activated prodrug to kill actively dividing (cancer) cells to neighboring cells. This occurs when cell killing metabolites created by the conversion of the prodrug to its toxic form in cells expressing the suicide enzyme are transferred to surrounding cells. This enhances tumor cell killing while eliminating the need for suicide gene delivery to every cancer cell. Thus, suicide gene therapy is hoped to be safer and more effective than current cancer treatment methods such as radiation therapy, chemotherapy, and surgery which have many downfalls. For example, radiation therapy and chemotherapy cause harmful side effects to healthy cells and tissues, and surgical resection of tumors often does not result in removal of all tumor cells.

While suicide gene therapy is promising, one current limitation is the low affinity of suicide enzymes towards their prodrug substrates such that insufficient amounts of toxic metabolites are produced for effective tumor ablation. Another problem is that suicide enzymes studied to date are foreign to the human body and may initiate an immune response in vivo that can prevent the full therapeutic effect of this approach. The overall goal of this study is to overcome these limitations by shifting the activity of the human deoxyguanosine kinase (dGK) enzyme from its natural substrate to the prodrug ganciclovir (GCV) for use in suicide gene therapy. Although dGK is currently not used as suicide enzyme because it has low activity towards GCV, we seek to create mutations in the enzyme at the active site to optimize its activity to the prodrug. Before mutagenesis studies can commence, our first
goal is to establish an easy and reliable system to screen thousands of dGK variants for dGK function and sensitivity to the prodrug, GCV.

A suitable screening system involves transforming *E. coli* with dGK variants and growing them on selective media that allows only cells expressing a functional dGK enzyme to be viable. Due to metabolic differences between *E. coli* and human cells, establishment of a dGK screening system is challenging. Three different approaches to develop a selection or screening system have been investigated. The first requires the construction of a genetically modified *E. coli* strain to select for dGK activity by direct genetic complementation. The other approaches are more indirect and involve the construction of fusion dGK enzymes in which another enzyme with an established selection system in *E. coli* is expressed together with dGK. The selection of the fused enzyme acts as a screening tool for full-length dGK expression rather than function.

The specific project goal is to develop a screening system of novel human suicide genes that can be used to improve treatment for cancer, a disease that affects millions of people. Once we establish a screening system for dGK mutants, we will use regio-specific random mutagenesis to create random mutations that could improve dGK's ability to convert GCV to its toxic form. The significance of this research is that the creation of novel dGK mutants has potential to not only improve the cell killing power of suicide gene therapy but also eliminate the concern of an immune response in the patient. Overall, this research will advance gene therapy through the development of a more potent and selective cancer treatment with decreased side-effects.
Acknowledgments:

I would like to acknowledge that this research was done with the assistance of Adam Johnson, and I would like to thank him for all of his help, patience, and support throughout the process. I would also like to thank everyone else in the Black lab who advised me during my research, including Stacy Hathcox and especially Dr. Margaret Black. Finally, thank you to the School of Molecular Biosciences and the STARS program for giving me the opportunity to gain valuable experience through research.
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Introduction:

Cancer is the second leading cause of death in the United States, and even with advancing cancer treatments, one in two males and one in three females are expected to develop cancer in their lifetime. It was estimated in 2010 that 1,529,560 men and women would be diagnosed with cancer and that 569,490 would die from the disease\[^{[1,2]}\]. Although widely used and accepted, current cancer treatments are invasive and have significant limitations. Most therapies combat cancerous cells using chemotherapy, radiation therapy, surgery, or a combination of the three, but such treatments are often ineffective and can cause modest to severe side effects like nausea, pain, and anemia\[^{[3]}\]. Further, many cancers metastasize making them inaccessible to surgical resection, and chemotherapy and radiation therapy demonstrate systemic toxicity and target all dividing cells, even healthy ones. Therefore, current research is investigating methods for safer cancer treatment with greater specificity and potency.

Suicide Gene Therapy

Suicide gene therapy is a particularly promising novel gene therapy option for cancer. Also known as gene-directed enzyme prodrug therapy (GDEPT), suicide gene therapy initially involves the delivery of a “suicide gene” which encodes an enzyme directly to tumor cells. Following suicide gene delivery and enzyme expression, a non-toxic prodrug is administered to the patient. A prodrug is an inactive drug that becomes functional when metabolized in vivo, and cell death occurs when the previously expressed enzyme converts the prodrug to its activated, or cytotoxic, form. Therefore, the prodrug initiates cell death exclusively in targeted tumor cells expressing a suicide enzyme—the basis of suicide gene therapy’s specificity.

Current delivery mechanisms include a variety of both viral and non-viral vectors, but these methods have demonstrated poor efficiency in delivering the suicide gene to tumor cells. While a large
part of research involving suicide gene therapy focuses on improvement of delivery mechanisms, researchers are also interested in exploiting the bystander effect as it eliminates the need for delivery of suicide genes to every tumor cell. The bystander effect is a crucial part of the suicide gene therapy process and allows for cytotoxic drug killing effects to be extended from suicide gene transfected cells to untransfected cells. Depending on the type of prodrug used, the cytotoxic metabolites are transferred between cells by gap junctions, apoptotic vesicles, or prodrug diffusion to create a localized anti-tumor toxicity that extends beyond individual cancer cells to eliminate all cancer cells\(^4\).

Many suicide enzyme/prodrug systems have been studied such as cytosine deaminase/5-fluorocytosine and deoxycytidine kinase/gemcitabine, but the suicide enzyme/prodrug system that uses thymidine kinase from Herpes Simplex Virus-1 (HSV-TK) as the suicide enzyme and ganciclovir (GCV) as the prodrug is the most widely studied and characterized suicide enzyme/prodrug system. It has demonstrated enough potential to be tested in 70 clinical trials, three of which have reached Phase III clinical trials since June 2007\(^5\). Although promising, the HSV-TK/GCV suicide gene therapy system still has limitations which include low affinity of the enzyme for its prodrug.

**Ganciclovir Activation Pathway**

GCV was originally developed as an antiviral agent against many viruses including Eptstein-Barr Virus, Cytomegalovirus, and Herpes Simplex Virus 1 and 2\(^6\). As seen in the pathway below, GCV is first converted to the monophosphorylated form by HSV-TK. The endogenous enzymes guanylate kinase (GMK) and nucleoside diphosphokinase (NDK) subsequently convert it to the diphosphate and triphosphate forms, respectively \(^7\). Cell death occurs when the triphosphate GCV metabolite directly competes with deoxyguanosine triphosphate and incorporates into DNA during DNA synthesis, leading to chain termination and cancer cell apoptosis. The efficacy of a suicide enzyme/prodrug system depends on overcoming the rate limiting step in the activation pathway which in this case is the
monophosphorylation of GCV by HSV-TK. Once phosphorylated, the GCV metabolites become charged, non-diffusible molecules. The bystander effect of this system, therefore, mainly relies on the transportation of cytotoxic metabolites, like GCV-triphosphate, to surrounding cells via gap junctions [8].

![Figure 1. GCV activation pathway. HSVTK, Herpes Simplex Virus thymidine kinase; GMK, guanylate kinase; NDK, nucleoside diphosphokinase; DNA POL, DNA polymerase](image)

Suicide gene therapy potency is limited largely by the low affinity of naturally occurring suicide enzymes for their synthetic prodrugs. Our lab focuses specifically on overcoming this limitation. We have performed studies previously to optimize suicide enzymes for greater activity to their respective prodrugs using random mutagenesis coupled with selection by genetic complementation in *E. coli*. The thymidine kinase gene from the Herpes Simplex Virus-1 has broad substrate specificity to thymidine and its analogs as well as guanosine analogs like GCV that facilitates the success of the HSV-TK/GCV system. In enzyme kinetics, the Michaelis-Menten constant, $K_m$, represents the inverse affinity of enzymes for their substrates. $K_m$ values for suicide enzymes in general are very high, and thus, high levels of prodrug dosages must be supplied for adequate tumor ablation. There is a limit to the effectiveness of prodrug dosage because, while by definition a prodrug like GCV is generally non-toxic at low levels, high levels of the drug can have a myelosuppressive effect and become highly toxic to organs [9,10]. To overcome this complication, current techniques aim to shift activity of the enzymes from their endogenous activity towards prodrug conversion. One such method is known as regio-specific random mutagenesis in which overlapping oligonucleotides are used to create mutations within a
targeted region of the suicide enzyme. Active mutants are then selected by positive selection in *E. coli* and later subjected to negative selection to identify mutants with increased prodrug sensitivity\[^{11}\].

Using this method, this lab has created HSV-TK mutants improved in converting GCV to its monophosphate form. Mammalian cancer cells transfected with one optimized mutant, the SR39 mutant of HSV-TK, when used in combination with GCV were shown to increase tumor cell killing compared to wild-type HSV-TK transfected cells. The improved activity of SR39 with the prodrug GCV can be attributed to regio-specific random mutagenesis targeting the active site resulting in five amino acid substitutions\[^{11}\]. These mutants illustrate the progress occurring in suicide gene therapy research, but the affinity of enzymes to their prodrugs is only part of the clinical limitations. Novel enzyme/prodrug systems are being investigated because suicide enzymes studied in suicide gene therapy to date are foreign to the human body and may elicit immune responses *in vivo*. This particular study is focused on generating a human enzyme with homologous activity to HSV-TK able to efficiently convert GCV to its monophosphate form.

**Deoxyguanosine Kinase (dGK) and GCV**

Deoxyguanosine kinase (dGK) is a mitochondrial enzyme found in human tissues, and it is involved in the salvage pathway of deoxypurine nucleosides for mitochondrial DNA synthesis. It has broad substrate specificity and is able to phosphorylate purines in this pathway. This enzyme currently has low activity towards GCV which precludes its potential as a suicide gene, but because GCV is an analog of deoxyguanosine (the natural substrate of dGK), the activity of dGK to GCV may be increased via regio-specific random mutagenesis\[^{7,12}\]. Thus, dGK is an attractive enzyme to target in mutagenesis studies, and this project aims to create a novel suicide enzyme/prodrug system using the human enzyme deoxyguanosine kinase (dGK) and prodrug GCV.
**Graft Versus Host Disease**

In addition to cancer treatment, the proposed novel human based enzyme/prodrug suicide gene therapy system may also be applicable in the treatment of graft-versus-host-disease (GVHD), a fatal condition that can occur as a result of bone marrow transplantations in which donor T-cells initiate an immunologic attack on the tissues and cells of the recipient (patient) [13-14]. If GVHD manifests itself, a prodrug can be administered so that the “silent” enzyme is able to destroy engrafted cells and abrogate GVHD. Thus, this research seeks to create a human enzyme with high activity towards a prodrug that can both increase tumor cell eradication without concern for an immunologic response in the treatment of cancer as well as act as a safety gene in the treatment of GVHD.

**Research Question:**

To improve the efficacy of suicide gene therapy and gain a deeper understanding of the mechanisms responsible for tumor cell killing, this study seeks to optimize activity of the suicide enzyme dGK to the prodrug GCV and thereby create a novel human based suicide gene therapy system. We hypothesize that by establishing a positive and negative screening system, we can isolate dGK mutants with improved specificity for the prodrug GCV for use in an improved suicide gene therapy system.

**Previous Studies and Experimental Design**

Positive selection in *E. coli* refers to the identification of a certain characteristic via growth on selective media. It is achieved by introducing a gene encoding a specific enzyme activity into a strain of *E. coli* engineered to not be viable on selective media unless that enzyme activity is provided
exogenously or replaced endogenously. As seen in Figure 2, negative selection identifies bacteria grown on positive selection plates that express enzymes with improved activity towards the prodrug. When transferred to GCV containing negative selection plates, they are able to convert the drug to its toxic form and lead to cell death. This research focuses on establishing the positive selection system. Negative selection will then be performed to identify bacteria containing functional mutants.

**Figure 2.** Positive and negative selection identify functional dGK mutants with improved cell killing ability in the presence of GCV.

Previous mutagenesis studies have been successful in improving suicide enzyme affinity for their prodrugs using positive selection followed by negative selection. *E. coli* was used because it is a well characterized bacterial model in all kinds of genomic studies, replicates efficiently, and provides a much simpler system in which to study genes than a eukaryotic or human cell. For example, to create and isolate the HSV-TK mutants explained previously, a genetic complementation system used the *E. coli* strain BL21(DE3) tk’. When these cells deficient in thymidine kinase were grown on minimal media, only those complemented by HSV-TK activity were viable. Negative selection of functional variants was then used to identify and isolate mutants demonstrating improved sensitivity towards GCV compared to the wild-type enzyme such as SR-39[11].
Additional HSV-TK studies in our lab have improved the rate of prodrug conversion using a pathway engineering approach. In pathway engineering, two contiguous enzymes in a metabolic pathway are combined into one functional fusion enzyme construct. The goal is to improve overall suicide gene therapy efficiency by overcoming limitations caused by rate-limiting steps. One fusion gene constructed in our lab encodes a functional GMK/HSV-TK fusion protein that was shown to be superior to HSV-TK alone in cell killing. IC50 values which describe the GCV concentration necessary to achieve 50% cell killing were reduced by 175 fold in rat C6 glioma cells and catalytic activity was shown to improve by nine fold as compared to HSV-TK alone[15]. Thus both mutagenesis and pathway engineering approaches have led to enhanced prodrug-mediated cell killing.

The specific goal of this project is to establish a screening system for functional dGK enzymes using positive and negative selection as a means to establish a novel human dGK/GCV suicide gene therapy system. Different techniques including genetic complementation and pathway engineering are employed, and this paper describes the methods and outcomes of three separate design schemes attempted to achieve our goal (Figure 3). However, because bacterial cells do not encode dGK, selecting for its function in the E. coli model is a more involved process than direct complementation of a gene deficiency.

The first approach (A) is a selection system for dGK that utilizes the E. coli KK446(DE3) that encodes a ribonucleotide reductase (RR) mutation making it highly sensitive to hydroxyurea (HU), a potent inhibitor of RR activity. RR is an essential enzyme in E. coli for deoxynucleotide and DNA synthesis. When RR is inactive, cells are not viable. However, Lactobacillus acidophilus R-26 uses a combination of enzymes to catalyze the equivalent reaction to that of RR in E. coli. These enzymes include thymidine kinase (TK), deoxyadenosine and deoxycytidine kinase (dAK/dCK), and deoxyguanosine kinase (dGK) [16]. Our approach involves replacing RR deficiency in KK446(DE3) by
cloning dAK/dCK from *L. acidophilus* R-26 and the human dGK as is illustrated in Figure 4 (TK is endogenous in *E. coli* and thus is not actually cloned into the cell). We anticipate that growth occurs in the presence of HU when all required enzyme functions are replaced in *E. coli* KK446(DE3).

**Figure 3.** Schematic outline of three designs tested to create a screening system for dGK mutants.
Once this genetic complementation system is established, we can mutate dGK using regio-specific random mutagenesis and screen for functionality by growth on media containing HU. Because KK446(DE3) cells encode TK endogenously and the project’s goal is to eventually improve the human dGK gene for activity towards GCV, the approach involves cloning dAK/dCK from *L. acidophilus* R-26 together with human dGK, the enzyme of interest in these studies. The combination of both enzyme activities alongside endogenous TK activity theoretically allows for the production of all deoxynucleotides, therefore allowing for genetic complementation and cell growth\[^{16}\].

The next approaches both involve creating a dGK fusion enzyme with enzymes to facilitate selection by complementation. The first approach (B) is to create a deoxyguanosine kinase (dGK)/guanylate kinase (GMK) fusion protein. GMK is the second enzyme in the GCV activation pathway, and the *E. coli* strain TS202A(DE3) is conditionally deficient in GMK activity. To allow for cell growth on selective media plates, GMK activity must be substituted in the cell by transforming a plasmid vector containing a functional GMK gene. The positive selection system utilizes a dGK enzyme with a stop codon inside of the active site to be mutated. Mutants can be identified by expressed protein because the stop codon has been removed allowing for complete read-through during translation.
Because the dGK and GMK proteins are attached in the fusion construct, growth of transformants on selection plates confirms restoration of an in-frame dGK gene. In this case, the gene encoding the GMK protein, \( gmk \), is subcloned adjacent to dGK from pETHT:gmk into pET23d:dGK creating the pET23d:dGK/gmk plasmid. The dGK gene in pET23d does not encode a stop codon so that the two genes are transcribed and translated together to create a single protein. Cells with GMK activity, and thus dGK expression, are able to grow on \( E. coli \) TS202A(DE3) selection plates establishing a system to identify transformants containing the dGK gene.

In the final design to select for functional dGK clones (C), we aim to create a fusion enzyme construct of dGK and bUPRT (bacterial enzyme uracil phosphoribosyl transferase). bUPRT is a pyrimidine salvage pathway enzyme that converts 5-fluorouracil to 5-fluorouracil monophosphate. Whereas the dGK/GMK protein has the potential to overcome rate limiting steps through pathway engineering as well as select for dGK, bUPRT’s purpose in this particular fusion enzyme is simply to be a screening tool for restoration of an in-frame dGK gene. In previous suicide gene studies involving fusion enzymes, a bUPRT deficient strain of \( E. coli \), BM604(DE3), was successfully utilized to select for UPRT activity\(^{[17]} \). In this experiment, the dGK/bUPRT construct is used to transform these cells. Only transformants able to confer bUPRT activity grew due to read-through from a restored dGK gene. Therefore, transformed cells expressing the entire dGK/bUPRT fusion construct grow on selective plates. Subsequent analysis of transformants identifies functional dGK variants with desired characteristics.

**Methodology:**

Because in principle the screening process acts to identify expression and activity of genes transformed into bacterial cells, a basic process is applicable in all three experimental schemes described above. As seen in the schematic diagram below (Figure 5), the DNA insert containing the gene of
interest is first isolated. Then, both the plasmid vector and the insert are digested using restriction enzymes, naturally occurring enzymes that cut DNA at certain sequences. A ligase enzyme is then used to rejoin the resulting linear DNA strands with the insert inside of the plasmid. Once the insert is ligated into the plasmid vector, competent cells are transformed. Following gene expression, protein and enzyme activity analysis are completed on the transformed cells.

Figure 5. The same basic process is used in each sub-project described for cloning genes into plasmid vectors. The dAK/dCK gene has to be purified from genomic DNA whereas the other genes are isolated from plasmids.
**Gene Isolation**

Before PCR amplification of the dAK/dCK gene, an additional step is necessary to purify *Lactobacillus acidophilus* R-26 genomic DNA whereas dGK, gmk, and bUPRT have previously been cloned into a bacterial expression plasmid or vector. To purify the genomic DNA, 75mL of Difco Lactobacilli MRS broth (Becton, Dickinson and Company) is inoculated with 300μL of an overnight *L. acidophilus* R-26 culture and incubated overnight at 37°C. The culture is centrifuged at 4000 RPM for 15 min, and cells are then resuspended in 5mL of lysis buffer (6.90g 50mM NaH₂PO₄, 17.54g 300mM NaCl, and .68g 10mM imidazole in 1L of H₂O) in a 37° water bath for 2hrs. 500μL of 10% SDS and 100μL of 25mg/mL proteinase K are then added and the solution is incubated in a 55° C water bath for 2hrs. One mL of the lysis solution is removed as crude lysate, while the remaining lysis solution is subsequently centrifuged and 1mL of the resulting supernatant containing genomic DNA is removed as cleared lysate. Chromosomal DNA is then subjected to PCR to amplify the specific gene of interest, dAK/dCK.

**PCR Amplification of dAK/dCK:**

First, the sequences encoding dAK/dCK are amplified by polymerase chain reaction (PCR) using primers designed in each experiment with unique restriction sites. DNA fragments are amplified in this process from a PCR reaction containing 5μL 10X reaction buffer, 5μL dNTP’s, 1μL (+) strand primer (10pmol/μL), 1μL (-) strand primer (10pmol/μL), 1-2μL DNA sample, 3μL 25mM MgCl₂, 0.5μL Taq polymerase, and up to a volume of 50μL with nuclease free H₂O. The starting PCR temperature is 95°C for 30sec followed by 30 cycles of 95°C for 30sec, 50°C for 1min, and 72° for 45sec followed by 72°C for 6min. Primers designed and used for each cloning project are described in Table 1.
Table 1. Primers used in PCR amplification of insert sequences throughout the project.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Scheme</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB 662</td>
<td>5' GGC GCT GCA GGC ATC TAT CTT ACA AGA TCA CC 3'</td>
<td>KK446(DE3) Genetic Complementation</td>
<td>(+) strand amplification of dAK/dCK encoding region of <em>L. acidophilus</em> R-26 with addition of <em>PstI</em> site.</td>
</tr>
<tr>
<td>MB 643</td>
<td>5' GCC GGG ATC CCA GCT GAT GCT GTA AGT GGT GC 3'</td>
<td>KK446(DE3) Genetic Complementation</td>
<td>(-) strand amplification of dAK/dCK encoding region from <em>L. acidophilus</em> with addition of <em>BamHI</em> site.</td>
</tr>
<tr>
<td>MB 687</td>
<td>5' GGC CGG ATC CGA TGG CAG GAC CTA GG 3'</td>
<td>dGK/gmk Fusion</td>
<td>(+) strand amplification of gmk from pET23d:gmk with addition of <em>BamHI</em> site.</td>
</tr>
<tr>
<td>T7 Terminator</td>
<td>5' TAT GCT AGT TAT TGC TCA G 3'</td>
<td>dGK/gmk Fusion, dGK/bUPRT Fusion</td>
<td>(-) strand amplification of gmk from pET23d:gmk, (+) strand amplification of bUPRT from pETHT:bCD/UPRT.</td>
</tr>
<tr>
<td>MB 712</td>
<td>5' GGC CGG ATC CGA TGG CTA AGA TCG TG 3'</td>
<td>dGK/bUPRT Fusion</td>
<td>(+) strand amplification of bUPRT from pETHT:bCD/UPRT with addition of <em>BamHI</em> site.</td>
</tr>
</tbody>
</table>

Following amplification, the PCR product contains millions of copies of each insert DNA flanked by unique restriction sites introduced by the designed primers. To prepare the DNA for ligation into the pHSG576 or pET23d:dGK vector depending on the project, these fragments are digested with restriction endonucleases corresponding to restriction sites found within the vector. The digestion reaction includes 45μL amplified insert DNA, 3μL restriction enzyme 1, 3μL restriction enzyme 2, 1μL BSA, 7μL reaction buffer, and 11μL nuclease free H₂O. Once mixed, the reaction is incubated for 2hrs in a 37° water bath. In order to troubleshoot various setbacks and obtain ideal ligation conditions, this
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protocol is varied slightly throughout the experiment. For example, the gel extraction of PCR amplified products can be completed before or after insert digestion, digestion reactions can be adapted for greater or lesser volumes of DNA, and incubation time can be varied.

**Preparation of pHSG576 and pET23d:dGK Plasmid Vectors**

Depending on the experimental design, pHSG576 or pET23d:dGK is prepared for cloning by digestion using the same restriction enzymes used to digest the corresponding PCR amplified product to be inserted. Reactions include 3μg plasmid DNA (pHSG576 or pET23d:dGK), 3μL restriction enzyme 1, 3μL restriction enzyme 2, 3μL 10X reaction buffer, 1μL BSA, and up to 30μL volume of H₂O. Once mixed, reactions are incubated in a 37° C water bath for 2hrs. Preparing vectors for cloning requires an additional step compared to insert preparation, however. To prevent re-ligation of the plasmid to itself and to increase the efficiency of the cloning reaction, digested plasmids are often treated with a phosphatase enzyme to remove the 5′ phosphate left on the DNA after being cut by an endonuclease. The free phosphate is needed for the ligation reaction but by removing it from the plasmid, the insert is more likely to be ligated to the plasmid than the plasmid is to re-ligating to itself. Vector digestion reactions thus undergo shrimp alkaline phosphatase (SAP) treatment in which the digestion reaction is first incubated in a 65°C water bath for 20min to heat inactivate the restriction enzymes; then 3μL SAP, 4μL SAP buffer (or enough to make 1X), and nuclease free H₂O needed for a total volume of 10μL are added to the reaction and incubated for an additional 1hr in a 37°C water bath. Following incubation at 37°C, the reaction is stopped by heat inactivation at 65° C for 20min.

**Ligation:**

Ligase is an ATP dependent enzyme and is responsible for repairing nicks in dsDNA. It uses a phosphate group left on the 5′ end of a cleaved piece of DNA to join two ends together. These cloning
experiments join the digested insert and vector using the T4 DNA ligase enzyme in a ligation reaction including 9μL of the restriction enzyme digested insert, 1μL of the restriction enzyme digested and SAP treated vector, 1μL T4 DNA ligase, and 1μL T4 DNA ligase buffer. Reactions are then incubated overnight in a 12°C water bath. A control reaction is made in the same way but replacing the insert with nuclease free H2O.

**Transformation by Electroporation:**

Electroporation uses an electric pulse to transform circular plasmid DNA into competent cells. Plasmids contain at least one antibiotic resistance gene, so the transformed bacteria are grown on antibiotic containing selective media so that only cells grow that contain circular plasmid DNA. For transformations, 1μL ligation (or control) mix is initially added to 40μL of competent cells. Cells are transferred to a cuvette and electroporated at 1400V for 5 msec. Bacteria are then resuspended in 1mL SOC solution (20g Bacto-peptone, 5g yeast extract, 10mL 1M NaCl, 2.5mL 1M KCl, 10mL 1M MgSO4, 10mL MgCl2, 36g glucose in up to 1L of H2O) and incubated in a 37°C shaker for 20-30min. Cultures are transferred to a 1.5mL Eppendorf tube and centrifuged for 1min at 13,300 RPM, and the resulting supernatant is discarded. Pellets are then resuspended in 100μL SOC and spread onto 2xYT+ carb plates (16g Bacto tryptone, 10g yeast extract, 5g NaCl, 15g Bacto agar, and 950mL H2O with 50μg/mL carbenicillin). 2xYT media plates are made selective by the addition of an antibiotic correlating to the plasmid transformed: 2xYT+ carbenicillin(carb) is used for pET vectors which confer resistance to carb while 2xYT+chloramphenicol (cam) at 20μg/mL is used for the pHSG576 vector which confers resistance to cam.

While colonies that grow on antibiotic containing media are expected to contain the insert that has been ligated into the plasmid vector, background colonies are sometimes present due to re-ligation
or incomplete vector digestion. Background levels are determined using an H₂O control in the ligation reaction.

**Analysis of Successful Cloning:**

To confirm successful cloning, all colonies are screened by retrieving the plasmid DNA from the bacteria using the boiling prep method, digesting the plasmid, and using gel electrophoresis to identify which colonies contain the insert.

**Boiling Prep Method:**

Plasmid DNA is purified from transformed cells using the boiling prep method. In this method, 1mL of overnight culture is spun down in a microcentrifuge tube. Culture supernatant is then aspirated off and the pellet is resuspended in 350µl boiling prep buffer (20mL 40% sucrose, 0.5mL Triton X-100, 10mL 0.5M EDTA pH=8, 0.5mL 2M Tris-HCl pH=8, in up to 100mL H₂O). Twenty-five µL of lysozyme from chicken egg white is added and the solution is mixed by inversion. Solutions are boiled for 40sec and spun for 8min at 13,300 RPM in the microcentrifuge. The pellet is then removed with a toothpick, and 40µL of 3M sodium acetate (pH 5.2) is added followed by 220µL 100% isopropanol. The solution is then mixed by inversion and incubated at room temperature for 5min. Each tube is spun for 5min and the supernatant is aspirated. Pellets are washed in 250µL of 70% EtOH, vortexed, and centrifuged for 4min. EtOH is then aspirated, and the remainder is allowed to evaporate by air drying. Finally, the DNA is resuspended in 40µL of 1xTE buffer (1:10 dilution in H₂O of 100mL 1M Tris-Cl pH=7.5 and 20mL .5M EDTA pH=8.0).

**Wizard Prep:**

Plasmid DNA is purified for sequencing using the Promega Wizard Plus SV Minipreps DNA Purification System following the Centrifugation Protocol. Cloning is later verified by sequencing.
Protein and Enzyme Activity Analysis:

SDS-PAGE:

After successful cloning is confirmed by restriction enzyme digestion and by sequencing, protein expression of the cloned gene is analyzed. First, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), a technique used to separate proteins by size and charge, is performed to identify the protein of interest in comparison to all proteins in the cell. It also isolates the protein of interest in preparation for an immunoblot using an appropriate antiserum. In this project, genes are cloned into plasmids containing T7 promoter driven expression. pET vectors are transformed into various strains of bacteria labeled (DE3), meaning that expression of transformed plasmids is controlled by IPTG (isopropyl-beta-D-thiogalactopyranoside) induced expression of the T7 RNA polymerase from the lac promoter. Transformants are grown with IPTG to induce expression of transformed genes. Cells are then boiled with SDS, an anionic detergent, and DTT, a reducing agent. Together, these chemicals unfold the proteins in the cells and surround them with negative charges. Similarly to gel electrophoresis, SDS-PAGE utilizes polyacrylamide gels to separate proteins by an electrical current through random pores. The unfolded, negatively charged proteins diffuse through the porous gel towards the positive charge at different rates based on size. Proteins are separated by their sizes and compared to a molecular weight standard after staining with Coomassie Brilliant Blue.

Immunoblot:

SDS-PAGE is also done in preparation for protein immunoblots that verify the presence of a specific protein in a cell. Following SDS-PAGE, the proteins are transferred to a membrane and probed using a primary antibody specific to the protein of interest. Bound proteins are visualized using the Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad). A secondary antibody containing an alkaline phosphatase conjugate binds to the primary antibody and accordingly to the protein of interest. In a
color reaction initiated by reagents provided in the kit, the alkaline phosphatase cleaves 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Cleaved BCIP forms a dimer in the presence of nitroblue tetrazolium (NBT) and becomes an indigo dye which stains the bands containing the protein of interest. Immunoblots of the successfully cloned transformants containing the dGK/GMK construct are performed in this study with anti-dGK and anti-GMK primary antibodies.

**dGK Enzyme Assay:**

To determine dGK activity, an enzyme assay is performed that determines dGK activity using a tritium labeled deoxyguanosine (\[^3H\]-dGuo) as a substrate. The reaction buffer for the assay includes 250mM Tris-HCl (pH 7.6), 25mM MgCl₂, 10mM DTT, 0.5% Triton X-100, and 2.5mg/mL BSA. The reaction includes 2\(\mu\)L of the reaction buffer, 1\(\mu\)L 50mM ATP, 1\(\mu\)L 100\(\mu\)M \[^3H\]-dGuo, 1-50\(\mu\)g dGK protein, and up to 10\(\mu\)L of H₂O and is incubated at 37°C for 20min. The reaction is then quenched with 30\(\mu\)L of ice cold water and heated for 2min at 95°C. 40\(\mu\)L of the reaction is blotted on Whatman De-81 filter discs, allowed to air dry, washed 3 times in 100mL of 4mM ammonium formate for 5min, and washed twice in 95% EtOH for 2min. After the filters are air dried, they are placed in a scintillation vial with 5mL of scintillation fluid and counted on the Packard 1900CA Liquid Scintillation Analyzer. This method allows us to measure counts per minute (CPM) to compare the functionality of the transformed dGK enzymes compared to wild-type dGK enzymes.

**Results:**

**A: E. coli KK446(DE3) Project**

This genetic complementation approach involves co-expressing dAK/dCK from *L. acidophilus* R-26 and human dGK from pET23d:dGK in *E. coli* KK446(DE3). The combination of both enzyme activities alongside endogenous TK activity would theoretically allow for the production of all
deoxynucleotides, therefore allowing for genetic complementation and cell growth\textsuperscript{[19]}. To create the genetic complementation system, pHSG576:dAK/dCK and pET23d:dGK are used to transform \textit{E. coli} KK446(DE3). Together with endogenous TK, RR activity is expected to be complemented in the presence of HU by the plasmid expressed enzymes. While the pET23d:dGK construct has been created previously, dAK/dCK is amplified from \textit{L. acidophilus} R-26 genomic DNA and cloned into the pHSG576 vector which confers resistance to cam.

Crude and cleared lysates from \textit{L. acidophilus} R-26 are used in a PCR reaction with primers MB662 and MB643 to amplify the dAK/dCK containing fragment from the genomic DNA. These primers have been designed to amplify a region of DNA made of \~1700bp flanked by \textit{PstI} and \textit{BamHI} sites (Figure 6). The DNA fragment and the pHSG576 plasmid vector are both digested with \textit{BamHI} and \textit{PstI} and ligated together by T4 DNA ligase. A control reaction is created by substituting H\textsubscript{2}O for the insert, and both the resulting pHSG576:dAK/dCK plasmid and control are transformed into \textit{E. coli} NM522 and grown on 2xYT+cam\textsubscript{20} plates.

\begin{figure}[h]
\begin{center}
\includegraphics[width=\textwidth]{figure6.png}
\end{center}
\caption{KK446(DE3) Project: Amplification the dAK/dCK gene. 1\% agarose gel. Lane1: \textit{\lambda/HindIII/EcoR1} marker. Lanes 2-9: the PCR product from the cleared lysate of \textit{L. acidophilus} R-26 culture is used to amplify the dAK/dCK gene with MB662 and MB643. The dAK/dCK fragment is present at \~1700bp and is gel extracted then ligated into pHSG576.}
\end{figure}
The success of the ligation is confirmed by purifying the plasmid using the boiling prep method and digesting out the inserted fragment using PstI and BamHI, the same enzymes used to previously insert the gene. Thus, if cloned successfully, the dAK/dCK fragment would be visible at ~1700bp in gel analysis. Plasmid DNA from transformants is first purified using the boiling prep method and is then digested using restriction enzymes BamHI and PstI. As seen in Figure 7, eleven samples contain pHSG576 plasmids with inserted dAK/dCK genes. Results are confirmed by sequencing.

Figure 7. KK446 (DE3) Project: Restriction Enzyme Analysis of pHSG576:dAK/dCK Transformants

BamHI and PstI are used to confirm successful cloning of the dAK/dCK gene into pHSG576. The digestion reaction includes 4µL boil prep purified plasmid DNA, 0.5µL BamHI, 0.5µL PstI, 0.2µL BSA, 1.5µL BamHI buffer, and 8.3µL nuclease free H2O incubated for 1hr in a 37°C water bath. Lanes 1 and 16: λ/HindIII/EcoR1 marker; Lanes 2-15, 17-26: Plasmid DNA from transformants digested by BamHI and PstI.

Once the dAK/dCK gene from L. acidophilus R-26 has been cloned into pHSG576, replacement of RR activity in KK446(DE3) cells is assessed in the designed genetic complementation system (Table
2. *E. coli* KK446(DE3) containing the previously cloned pHSG576:hTK2 and *E. coli* KK446(DE3) containing pHSG576:dAK/dCK are transformed with 1μL pET23d:dGK or 1μL pET23d as a control and grown on dGK selection plates. Transformants grown on selective dGK media are resuspended in a 4% saline solution instead of SOC to ensure that no growth occurs with assistance from nutrients in SOC media. Human thymidine kinase 2 (hTK2) is a salvage pathway enzyme with activity towards deoxypyrimidine nucleosides that, along with dGK, helps provide all deoxynucleotides necessary for DNA synthesis in the mitochondria. The combination of this enzyme along with dGK is used as an alternative genetic complementation system. Both combinations of enzymes are tested to look for genetic complementation of replaced RR activity in the presence of dGK. Cells grew on nonselective 2xYT and selective dGK plates containing appropriate antibiotics but not on dGK selection plates containing HU. These results suggest that the genes can be cloned successfully into *E. coli* KK446(DE3) but do not replace RR activity. Genetic complementation is not achieved when pET23d:dGK is used to transform pHSG576:hTK2 or pHSG576:dAK/dCK (KK446(DE3)). Thus, we are not pursuing this design as a screening system for dGK.

### Table 2. *E. coli* KK446(DE3) Project: Genetic Complementation Test on dGK Selection Plates

<table>
<thead>
<tr>
<th></th>
<th>pHSG576:hTK2 (KK446(DE3))</th>
<th>pHSG576:dAK/dCK (KK446(DE3))</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET23d</td>
<td>Growth</td>
<td>Growth</td>
</tr>
<tr>
<td>pET23d:dGK</td>
<td>Growth</td>
<td>Growth</td>
</tr>
<tr>
<td>2xYT+carb50+cam20</td>
<td>Growth</td>
<td>Growth</td>
</tr>
<tr>
<td>dGK selection</td>
<td>Growth</td>
<td>Growth</td>
</tr>
<tr>
<td>dGK+HU</td>
<td>No growth</td>
<td>No growth</td>
</tr>
</tbody>
</table>
B: Guanylate Kinase (GMK) Fusion Protein Project

To create the dGK/GMK fusion protein, GMK is sub-cloned from the pET23d:gmkΔBamHI plasmid with the BamHI site removed from the multiple cloning region into pET23d:dGK. The purpose for removing the BamHI site from the plasmid containing gmk is to use BamHI as a unique restriction site in the cloning. The gmk insert is prepared by PCR amplifying the pET23d plasmid DNA with MB687 and T7 Terminator primers. The PCR product produces an ~800bp band that is gel extracted and prepared for insertion using BamHI and NotI. The pET23d:dGK plasmid vector is also prepared for cloning using the same restriction endonucleases as well as SAP treatment. The plasmid and insert are then ligated together and transformed into NM522 cells as described. A ~600bp band following digestion with EcoRI and NeoI is the expected product of the dGK/GMK fusion gene cloning (Figure 8). DNA from positive constructs is sequenced.

After successful cloning of gmk into pET23d:dGK is confirmed, the pET23d:dGK/gmk construct is transformed into E. coli TS202A(DE3), a kanamycin resistant strain of bacteria. In the absence of arabinose, GMK activity must be replaced for the cells to grow. Results show that the TS202A(DE3) selection plates work correctly as cells transformed with pET23d, pET23d:dGK, pET23d:gmk, and pET23d:dGK/gmk all grow on plates with arabinose while only pET23d:gmk and pET23d:dGK/gmk grow on plates without arabinose (Figure 9). TS202A(DE3) cells containing the pET23d:dGK/gmk construct are then grown and analyzed via SDS-PAGE and immunoblot using anti-dGK and anti-GMK antibodies (Figure 10). The dGK protein is ~30kDa while the GMK protein is ~23kDa. Results from the SDS-PAGE indicate that all three proteins are present (dGK, GMK, and dGK/GMK) at the correct sizes. When a dGK enzyme assay is performed, results indicate that the dGK is not active in the fusion construct (Figure 11). One possibility is that the fusion protein is not folding properly and dGK activity is impaired.
Figure 8. dGK/GMK Project: Restriction Enzyme Analysis of pET23d:dGK/gmk Transformants

Plasmids from transformants are purified using the boiling prep method. Then, they are digested with EcoRI and NcoI. Positives are expected to produce a band at ~600bp. Positives are confirmed by sequencing.

Figure 9. dGK/GMK Project: TS202A (DE3) Selection Plates

E. coli TS202A(DE3) containing plasmids pET23d, pET23d:gmk, pET23d:dGK, and pET23d:gmk/dGK are streaked onto TS202A(DE3) selection plates with and without arabinose. While all bacteria grow on the plate with arabinose, only pET23d:gmk and pET23d:dGK/gmk grow on plates without arabinose. This is expected and indicates that the plates select for GMK function.
Figure 10. dGK/GMK Project: Immunoblot Analysis

a.) An SDS-PAGE of BL21(DE3) tk' cells transformed with (Lanes 2-5) pET23d, pET23d:dGK, pET23d:gmk, and pET23d:dGK/gmk is performed using a Coomassie blue stain. Lane 1 contains a pre-stained SDS-PAGE low range marker. b.) Immunoblots are then performed using 1:40,000 anti-dGK and 1:10,000 anti-GMK primary antibodies. Visible bands correspond to predicted molecular weights of the proteins.
A dGK enzyme assay using $[^3]H$-dGuo as the substrate was performed for cells transformed with pET23d, pET23d:dGK and pET23d:dGK/GMK. Data using 0.5μg and 1 μg of purified protein is shown.

C. dGK/bUPRT Fusion Construct

In the final design for a screening system, we are creating a fusion enzyme with dGK and bUPRT, an enzyme used previously as a fusion construct. As seen in Figure 12, only *E. coli* BM604(DE3) with replaced bUPRT activity grow on selective media. The primer MB712 (Table 1) has been designed to bind to the bUPRT fragment of pETHT:bUPRT while adding a *BamH*I site. Together with the T7 Terminator primer, the fragment is amplified using the same PCR program described previously. Both the bUPRT fragment and the SAP treated pET23d:dGK are digested with *BamH*I and *NotI* and ligated together. The transformed plasmids are digested with *NcoI* and *EcoRV* so that a ~1000bp band is expected to be visible by gel analysis. Transformants with expected results appear to be present in one trial (data not shown), but sequencing produces negative results. The false positives seen on the gel are probably due to background from the pETHT:bUPRT plasmids used for insert...
amplification that have contaminated the experiment. We are currently altering digestion and ligation conditions to find the best conditions for successful cloning.

![Image of two Petri dishes](image.png)

**Figure 12. dGK/bUPRT Project: bUPRT Selection in *E. coli* BM604(DE3)**

The selection system for the pET23d:dGK/bUPRT system is demonstrated in BM604(DE3) cells transformed with pETHT and pETHT:bUPRT.

**Discussion:**

Human dGK has the potential to overcome limitations of current suicide gene therapy approaches and establish such therapy as a valuable tool in treating cancer as well as GVHD. The enzyme has broad substrate specificity as it is responsible for phosphorylating all deoxypurines in the salvage pathway for mitochondrial DNA synthesis. Additionally, GCV is an analog to guanosine, dGK's natural substrate. Human dGK, therefore, is a prime candidate for regio-specific random mutagenesis studies to create a suicide enzyme with improved activity towards GCV. Many downfalls with current suicide gene therapy systems' ability to effectively ablate tumor cells *in vivo* arise from using foreign enzymes in the human body. A human enzyme would not cause such an immune response,
and an improved dGK may also be used to treat GVHD if it arises in patients who have received a bone marrow transplantation.

The goal of this project is to set the groundwork for such studies by establishing a screening system for dGK mutants with improved activity towards the prodrug GCV using positive and negative selection. Because dGK is not a bacterial enzyme, the challenge is to develop such a system able to screen for dGK activity in *E. coli*. While results are not yet complete, three separate schemes have been designed and tested (Figure 3).

Design A is a genetic complementation system in *E. coli* KK446(DE3) which aims for direct complementation of RR activity in the presence of HU by cloning in several genes necessary in *L. acidophilus* R-26 for activity equivalent to that of RR. Although successful cloning of dAK/dCK and dGK has been achieved in KK446(DE3) cells, the genetic complementation system is not successful as seen in Table 2. While this is the most direct method to screen for dGK activity, complementation of such a complex system is difficult, and we are pursuing more indirect approaches as well.

Designs B and C both involve complementation associated with other enzyme activity by creating fusion enzyme constructs. The screening system identifies the accurate read-through of the dGK gene by the presence of the other enzyme in the construct. The first enzyme tested is the dGK/GMK fusion in design B. Although results show that the dGK gene is successfully cloned into *E. coli* TS202A(DE3) and GMK is active, the enzyme assay indicates that dGK is not active (Figures 10 and 11). Although dGK activity has not yet been observed in preliminary assays in the dGK/GMK fusion enzyme, results for wild-type dGK are not as expected. The enzyme assay method may not be accurate and this experiment will be repeated in the future. Another future direction in the dGK/GMK design is to clone a poly-glycine linker in-between the dGK and GMK in the construct. Adding a nonreactive string of amino acids between the two functional enzymes has been used previously to help
proper protein folding and achieve activity of both enzymes. The pathway engineering approach of creating a fusion dGK/GMK enzyme has potential in that GMK is able to be selected for in \textit{E. coli} TS202A(DE3) when grown on selective media. The dGK and GMK are joined together in a fusion construct, and thus the selection of GMK translates to the selection of dGK. Additionally, dGK and GMK are the enzymes that would carry out the rate limiting steps of the GCV activation pathway. In a fusion protein, the efficiencies of both enzymes acting subsequently on a substrate are increased and can be selected for.

In design C, a fusion enzyme construct of dGK/bUPRT is constructed and cloned into BM604(DE3) cells. Growth on selective media indicates read-through of the dGK gene. This cloning project has yet to be completed because the initial step of sub-cloning bUPRT into pET23d:dGK is proving difficult. This could be due to a number of reasons including incomplete digestion of the vector and insert or a high amount of re-ligation of the digested vector. This approach is also the least specific for dGK since the enzyme selected for, bUPRT, is not involved in the GCV activation pathway. However, bUPRT has been used before as a fusion enzyme in a similar study and so has potential for facilitating dGK screening. The dGK/bUPRT fusion enzyme remains a potential screening method, and digestion and ligation conditions are currently being altered to successfully sub-clone bUPRT into pET23d:dGK.

If we find that using genetic complementation to create a screening system for dGK is unfeasible, our focus will shift to structure-function studies of the dGK enzyme. We will compare the protein sequences of HSV-TK, another salvage pathway enzyme that has been shown to have activity towards GCV. We will then use random and site-directed mutagenesis to mutate residues in dGK and test the new variants for enzyme activity to GCV as well as thymidine (TK's natural substrate) in hopes
of identifying important residues in the active site that will advance our understanding of the mechanism needed to convert GCV to its monophosphate form.

**Conclusion**

We hypothesize that by creating a screening system for dGK mutants, we can use regio-specific random mutagenesis to improve the enzyme’s tumor cell killing potential in suicide gene therapy with GCV. Once such a mutant is created, future studies will investigate how a mitochondrial suicide enzyme affects the bystander effect and suicide gene therapy as a whole. Developing a screening system for dGK is proving more challenging than expected, and reasonable steps are being taken to overcome the difficulty of selecting for enzyme activity in *E. coli*, an organism that does not naturally express dGK.

In conclusion, suicide gene therapy has potential to be an improved cancer treatment if its current limitations can be overcome, such as the low affinity of currently used suicide enzymes for their prodrugs and the risk of an immunogenic response in patients due to use of enzymes foreign to the human body. This research aims to overcome these limitations by shifting the use of foreign enzymes in suicide gene therapy systems to mutagenized human enzymes with improved ability to convert the prodrug to its cell killing form. Such an enzyme that does not pose a risk of initiating an immune response in the patient has an added application in treating GVHD. The overall goal is to establish suicide gene therapy as a treatment option with increased potency targeted directly to cancer cells and reduced systemic toxicity in the patient compared to currently used cancer treatments such as radiation therapy and chemotherapy.
References:


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