EVALUATION OF THERMOSTABLE YEAST CYTOSINE DEAMINASE AND HERPES SIMPLEX VIRUS THYMIDINE KINASE FUSIONS IN DOUBLE SUICIDE GENE THERAPY FOR CANCER

By

MARILYN SANCHEZ-BONILLA

A thesis submitted in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE IN PHARMACOLOGY AND TOXICOLOGY

WASHINGTON STATE UNIVERSITY
Division of Pharmacology and Toxicology

DECEMBER 2009

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of MARILYN SANCHEZ-BONILLA find it satisfactory and recommend that it be accepted.

_____
Margaret E. Black, Ph.D., Chair

_____
Lisa M. Gloss, Ph.D.

_____
Nancy S. Magnuson, Ph.D.

_____
Arash Hatefi, Ph.D.

EVALUATION OF THERMOSTABLE YEAST CYTOSINE DEAMINASE AND HERPES SIMPLEX VIRUS THYMIDINE KINASE FUSIONS IN DOUBLE SUICIDE GENE THERAPY FOR CANCER

Abstract

by Marilyn Sanchez-Bonilla, M.S.
Washington State University
December 2009

Chair: Margaret E. Black

Suicide gene therapy is a promising alternative treatment for cancer that specifically targets tumor cells for destruction by delivering a gene encoding a suicide enzyme that is able to convert an anticancer prodrug to a cytotoxic product. Two widely studied and clinically used enzyme/prodrug systems are cytosine deaminase (CD) with 5-fluorocytosine (5FC) and Herpes Simplex Virus thymidine kinase (HSV-TK) with ganciclovir (GCV).

Both enzyme/prodrug systems have shown positive results in clinical studies but still exhibit limitations such as low transduction efficiency due to current delivery systems and low enzyme activity towards the prodrug. An approach to overcome the latter limitation is to create a fusion of both CD and HSV-TK in what is known as double suicide gene therapy (DSGT). DSGT takes advantage of two enzyme/prodrug systems to create a synergistic cytotoxic effect with the lowest prodrug doses possible. DSGT studies with bacterial CD (bCD) and TK fusion started in the late 1990s and showed enhanced tumor growth inhibition and radiosensitization following 5FC and GCV

treatment. 5FC effect on deoxynucleotide pools by allosteric regulation of the enzyme ribonucleotide reductase was suggested as the cause of the synergistic effect observed in bCD/TK. In contrast to bCD/TK studies, synergistic experiments are yet to be done for yCD/TK fusions. Previous studies in our laboratory have created CD and TK mutants (yCD_{double}, yCD_{triple} and SR39, respectively), with either improved thermostabilization or improved prodrug activity. These mutants were created using computational design (yCD) or regio-specific random mutagenesis (TK). As CD mutants and SR39 mutant showed improved activity and/or a greater tumor killing efficiency in comparison to wild type enzymes, we sought to evaluate the synergistic effect of mutant fusion enzymes. Even though yCD/SR39 fusions have been used with positive results in clinical trials, we hypothesize that by using thermostabilized yCD in fusion with SR39 a greater killing effect could be obtained.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

CHAPTER ONE

INTRODUCTION

According to the American Cancer Society, cancer is the second leading cause of adult death in the United States after heart disease. It is estimated that in 2009 alone 1.5 million new cases will be diagnosed and approximately 512,340 million will die of cancer which is roughly 1,400 deaths per day. Current cancer treatments include surgery, chemotherapy and radiotherapy. Even though standard treatments are still widely used, each approach has its problems and limitations. For example, although surgery is most commonly used to remove solid tumors, there is a high probability of incomplete tumor removal posing a high risk of relapse. While chemotherapy and radiotherapy kill dividing cells including both normal and tumor cells they often cause side effects such as nausea, vomiting, fatigue and loss of appetite. The possibility of resistance to chemotherapeutic agents may also occur because of cellular changes that could affect the amount of drug getting in or out of the cell (1). In addition to these side effects, patients often develop secondary forms of cancer, such as leukemia, after chemotherapeutic and radiation treatments have ended (2-6).

To overcome the limitations seen in standard treatments it is desirable to create a therapy that will target tumor cells while sparing normal cells from damage. Suicide gene therapy is an alternative approach to cancer treatment developed about 20 years ago. The advantage of suicide gene therapy resides in its ability to specifically target tumor cells for destruction by the delivery of a gene encoding a suicide enzyme. A schematic representation of suicide gene therapy and how it works can be seen in

Figure 1.1. Suicide gene delivery can be done either via viral or non-viral vectors which differ in their immunogenicity and transduction efficiencies. Viral vectors include retrovirus, adenovirus, herpes simplex virus, lentivirus and vaccinia virus. Even though viral vectors are characterized of having high efficiency in gene delivery their use is limited by the DNA loading capacity, toxicity and immunogenicity. Non-viral vectors such as naked DNA, liposomes, polymers and peptides are less toxic and less immunogenic but lack the gene delivery efficiency viral vectors possess. A review of the different kinds of gene delivery methods and their potential for cancer gene therapy can be found in Hatefi and Canine (2009) (*7*) .

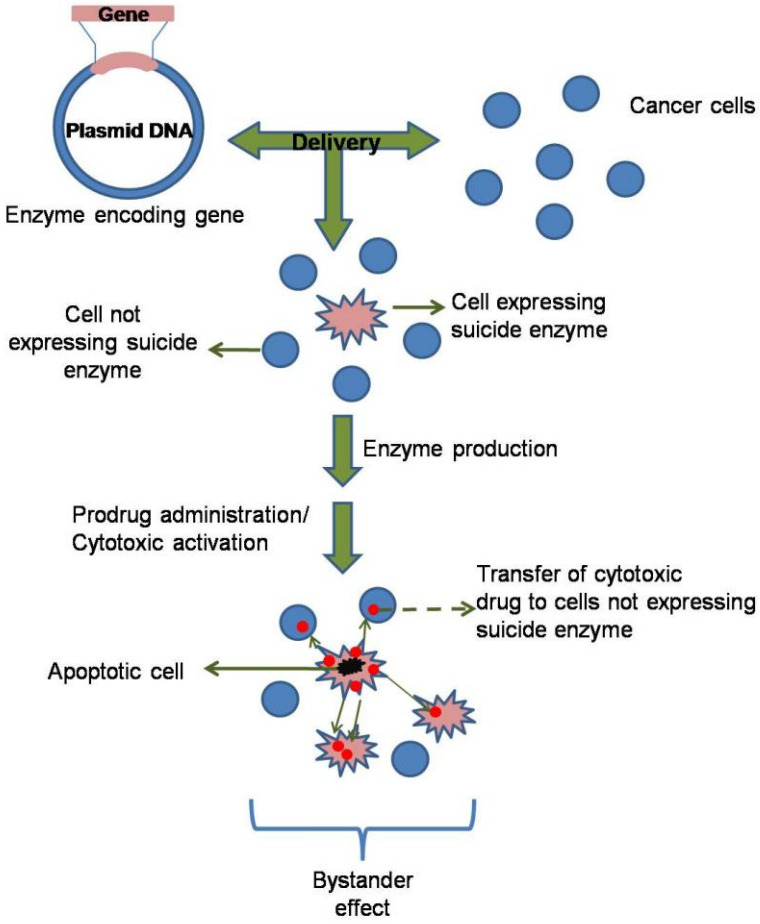


Figure 1.1. Schematic representation of suicide gene therapy.

Following successful gene delivery, the suicide enzyme is subsequently produced and in turn converts an administered non-toxic prodrug into an active toxic metabolite that induces apoptosis in transfected cells. This apoptotic effect may also be transferred to neighboring non-transfected cells by a phenomenon known as the bystander effect. The bystander effect by definition is the extension of killing effects from transfected cells to neighboring non-transfected cells via diffusion (8, 9), gap junctions (10-12) or apoptotic vesicles (13). Because of low cell transduction efficiencies observed in current delivery systems, a strong bystander effect is crucial to obtain total tumor regression. Although suicide gene therapy has its advantages and potential for cancer treatment, another key limitation is the low enzyme activity displayed toward the prodrug.

Ideally, a suicide enzyme suitable for suicide gene therapy protocols should possess several important characteristics (14). Firstly, it should have high catalytic activity towards a prodrug to be able to discriminate the enzyme's natural substrate which could be present in normal cells decreasing treatment efficacy. Secondly, to avoid damage to normal cells the suicide enzyme delivered to tumor cells must be different from endogenous enzymes. Finally, the suicide enzyme should be expressed in sufficient concentrations to be of clinical value. However, in reality, enzymes often have greater activity towards their natural substrates and poor activity towards a prodrug, thus requiring high doses of prodrug resulting in side effects. In addition, as the enzyme in most cases is foreign to the host, an immune response could be triggered which, depending on the situation, could be a positive event. Induction of an immune response could eliminate remaining tumor cells but if induced too early could

destroy the vector carrying the suicide gene thus preventing gene delivery. To overcome poor enzyme mediated prodrug activation we seek to improve suicide enzymes in term of stability and activity towards their respective prodrugs.

Our laboratory focuses on optimizing enzymes through enzyme and pathway engineering to obtain enzymes with higher sensitivity towards a prodrug for suicide gene therapy (15-19). Several mutagenesis approaches may be used to achieve enzyme improvement (15). One approach is site-directed mutagenesis where a single amino acid is changed, however, this method has a low success rate and does not usually provide variants with significantly improved activity. Another approach, random mutagenesis, targets specific regions either in or out of the active site for amino acid substitutions that might switch substrate specificity by favoring a prodrug over the enzyme's natural substrate. Regio-specific random mutagenesis uses two or more overlapping oligonucleotides, with at least one containing a randomized sequence for the target region of interest within the gene(Figure 1.2) (20). The first and last oligonucleotide used must contain a restriction site at their 5'-end for cloning purposes. Following annealing, extension and amplification, a randomized gene pool is obtained and then cloned into a "dummy" vector. A "dummy" vector is created by introduction of a stop codon that inactivates our gene of interest which is expressed in a bacterial expression vector. Insertion of randomized fragments restores the enzyme open reading frame. The randomized DNA pool is then transformed into a suitable host that can be used in a genetic complementation system. Finally, improvement can also be done using computer modeling programs to identify sequences of interest that might alter the activity of an enzyme.

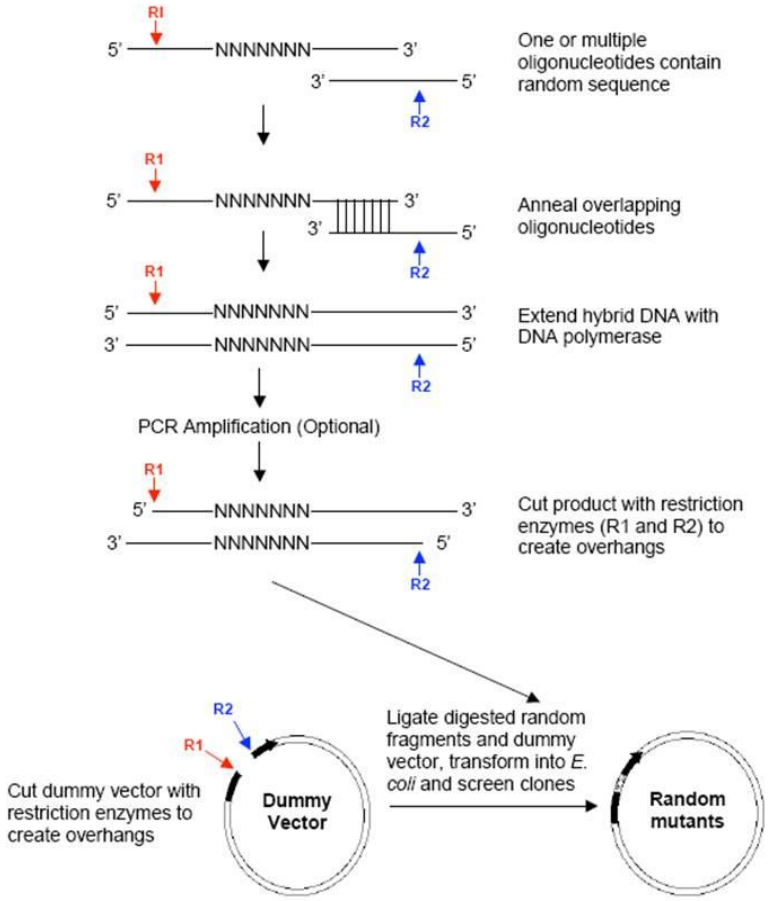


Figure 1.2. Overview of regio-specific random mutagenesis. N: randomized residues; R: restriction enzyme.

Herpes simplex virus thymidine kinase/Ganciclovir (HSV-TK/GCV)

Two widely studied and clinically used gene/prodrug systems are Herpes Simplex Virus Thymidine Kinase (HSV-TK) with ganciclovir (GCV) and Cytosine Deaminase (CD) with 5-fluorocytosine (5FC) (21, 22). Thymidine kinase (EC 2.7.1.21) is an important enzyme in nucleotide metabolism and is responsible for the conversion of thymidine to dTMP by the transfer of the γ-phosphate from ATP to thymidine. Unlike human thymidine kinase, HSV-TK has broad substrate specificity and is able to phosphorylate pyrimidines (thymidine and deoxycitidine), pyrimidine analogs

(azidothymidine and brivudin) and guanosine analogues (ganciclovir, acyclovir, buciclovir, and penciclovir).

HSV-TK, a dimer of 45kDa subunits, catalyzes the initial and rate limiting phosphorylation of the guanosine analog GCV to its monophosphate form (Figure 1.3). The monophosphorylated GCV is then further phosphorylated to its cytotoxic GCV-TP form by endogenous enzymes before being incorporated into DNA where it causes double strand destabilization and subsequently, cell death. GCV has been used since early 1980s for the treatment of cytomegalovirus (CMV) infections, a member of the *herpesviridae* family. It was not until 1986 that Moolten first suggested GCV be used in combination with HSV-TK for the treatment of cancer as HSV-TK exhibits 1000-fold greater activity towards GCV than endogenous cellular thymidine kinases (21, 23). Although GCV-TP is the predominant toxic form of the prodrug, the exact mechanism of GCV-mediated toxicity in cancer cells is less well understood than acyclovir (ACV), also a guanosine analog (Figure 1.3). Originally, the anti-herpetic drug ACV was considered a more suitable prodrug because of its low toxicity (15). Because of the low activity HSV-TK displays for ACV ($K_m \approx 400\mu M$) it would require very high concentrations of ACV to see any effect in tumor burden. The K_m value of HSV-TK for GCV is approximately 47µM which is 8-fold lower than that of ACV making GCV a better substrate (15). ACV has one hydroxyl group in its acyclic ‘sugar’ while GCV has two hydroxyl groups allowing for both to be incorporated into DNA causing immediate chain termination during synthesis. The lack of a complete sugar ring makes the triphosphate form of the prodrug a poor substrate for DNA synthesis causing DNA destabilization upon its incorporation by DNA polymerase (24).

The main bystander mechanism of GCV requires direct cell to cell contact via gap junctions and/or apoptotic vesicles for the transfer of toxic metabolites to neighboring non-transfected cells (13, 25, 26). Gap junctions are protein channels in cell membranes that allow the passage of ions or small molecules from cell to cell. It has been shown that connexin-mediated gap junctional intercellular communication enhances the bystander effects in cells expressing HSV-TK (11, 27). Apoptotic vesicles or apoptotic bodies are cellular debris that arises when cells undergo apoptosis and can be recognized and engulfed by non-transfected neighboring tumor cells. The process in which these untransfected cells take up apoptotic vesicles was suggested to be by phagocytosis (13).

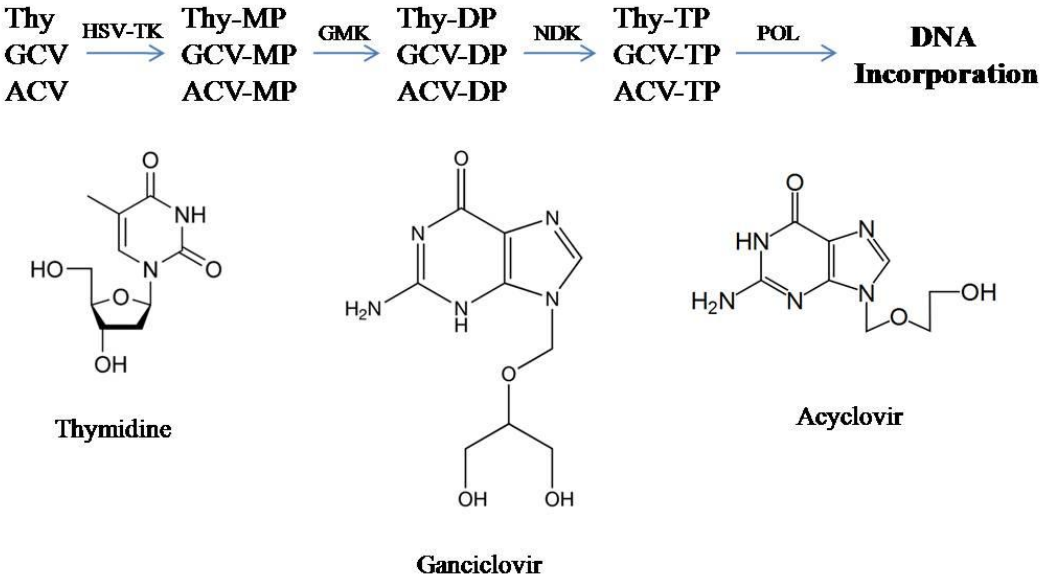


Figure 1.3. GCV activation by HSV-TK. HSV-TK is responsible of the initial phosphorylation of GCV which is then converted to the di- and the tri-phosphate forms by cellular GMK and NDK, respectively. The cytotoxic GCV-TP is then incorporated into DNA by DNA polymerase (POL), destabilizing the chain and terminating synthesis, causing cell death. (HSV-TK: Herpes simplex virus-thymidine kinase; GMK: guanylate kinase; NDK: nucleoside diphosphokinase; POL: polymerase; Thy: thymidine; GCV: ganciclovir; ACV: acyclovir; -MP: mono-phosphate; -DP: di-phosphate; -TP: tri-phosphate)

Improving Thymidine Kinase

Although GCV is the current prodrug of choice for suicide gene therapy in combination with HSV-TK, there are still limitations with the use of this prodrug. The K_m value of HSV-TK for GCV is approximately 100-fold higher than its K_m value for thymidine (K_m = 47μM versus 0.5μM) (15). Because of the poor activity displayed by HSV-TK towards GCV, prodrug doses administered to cancer patients are high and may result in life-threatening side effects such as bone marrow depression or myelosuppression (28). Myelosuppression is a condition that can range from mild to life-threatening in which the bone marrow is unable to produce red blood cells, white blood cells and platelets that can lead to anemia, an increased risk of infection, or bleeding. Extensive work has been done to create improved HSV-TK variants with increased activity towards GCV so that lower doses could be used to obtain effective tumor ablation with reduced side effects (18, 29-31).

Before the structure of HSV-TK was known, it was suggested that amino acid residues in proximity to two highly conserved sites known to play an important role in substrate binding would be suitable targets for regio-specific random mutagenesis(32). After the pool of random mutants was created, DNA was transformed into a suitable *E. coli* strain (BL21(DE3)*tdk*$^-$) and plated onto rich media plates to ascertain the number of transformants. To identify variants with improved activity, a genetic complementation system was used that aims to identify variants that are both TK active and GCV sensitive and can be divided into two steps: positive and negative selection. As the *E. coli* strain used was thymidine kinase (*tdk*) deficient, only transformed cells possessing TK activity can complement the deficiency and therefore grow on selection plates

containing thymidine as the sole pyrimidine source. In the negative selection step, TK active variants from positive selection plates are transferred to negative selection plates containing thymidine and GCV. Variants that show improved activity are unable to form colonies at lower concentrations of GCV than allow the wild type enzyme to grow.

In a previous study a DNA library of more than a million transformants was obtained and processed through genetic complementation with only a handful showing TK activity and a few with significant improvement in terms of GCV sensitivity in comparison with wild type HSV-TK expressing cells (18, 29, 32). One TK mutant that proved to be exceptional in terms of activity towards GCV is SR39. This mutant has five amino acid substitutions (L159I, I160F, F161L, A168F, L169M) and exhibits high GCV and ACV sensitivity in rat C6 glioma cells *in vitro*, with IC_{50} values of 0.017 µM and 0.11 µM respectively. These studies translated to a 294- and 182-fold reduction in IC_{50} compared to wild type HSV-TK for GCV and ACV, respectively. *In vivo* xenograft tumor studies demonstrated that SR39 was better at inhibiting tumor growth requiring a 10-fold lower GCV concentration (0.5 mg/kg) needed to see an effect in tumors expressing wild type TK (5.0 mg/kg) (18). Low GCV doses needed to prevent tumor growth in xenograft tumor models with SR39 demonstrated a significant decrease to current doses used in most animal experiments (up to 300 mg/kg/day) (28). In another study where immuno-deficient (SCID) mice were injected with metastatic prostate CL1 cells expressing either wild type TK or SR39 and treated with 20 mg/kg GCV, SR39 demonstrated a 63% reduction in tumor growth in comparison with tumors expressing wild type TK (33). In addition to rat C6 glioma cells, SR39 has also been shown to be effective in increasing cell killing in various cell lines following treatment with GCV

including: human mesothelioma (I-45, REN, LRK, H513, H2052), human non-small cell lung cancer (A549), cervical carcinoma (C33A) and mouse fibroblast tumors (EJ62)(34). In bystander experiments, rat C6 glioma cells transfected with empty mammalian expression vector (pUB) were mixed at different ratios with cells expressing SR39 and treated with 80 µM GCV. Mutant SR39 displayed stronger bystander effect requiring only 20% of cells expressing enzyme to obtain 50% cell killing as opposed to wild type TK where no bystander effect was seen (35). The improvement of HSV-TK activity towards GCV through the creation of SR39 has opened a number of possibilities for its use, not only as an excellent suicide enzyme for suicide gene therapy protocols but also as a reporter gene for positron emission tomography (PET) imaging (36-40). Positron emission tomograpy is a non-invasive nuclear imaging technique that produces a three-dimensional image and that can be used to monitor gene delivery, gene expression and cancer therapy efficiency (40). SR39 has proven to be a powerful reporter protein as it can phosphorylate a number of radiolabeled substrates used as reporter probes that includes both pyrimidine (uracil-based probes) and purine (acycloguanosine-based probes), while HSV-TK wild type has higher activity for pyrimidine-based probes (41). Following initial phosphorylation by HSV-TK, radiolabeled compounds cannot diffuse from cell to cell and thus allows an entrapment and accumulation of product within the cells.

Cytosine Deaminase/5-Fluorocytosine (CD/5FC)

A second system that is widely used for suicide gene therapy is cytosine deaminase (CD)/5-fluorocytosine (5FC). Cytosine deaminase (EC 3.5.4.1) is an enzyme in the pyrimidine salvage pathway and is present in bacteria and fungi but not

in mammalian cells. CD catalyzes the deamination of cytosine to uracil, and also catalyzes the hydrolytic deamination of the anti-fungal agent 5FC to its active form, 5-fluorouracil (5FU), a widely used chemotherapeutic agent (Figure 1.4) (17). 5FU, developed in the 1950s as an anticancer agent, is a uracil analog and has been shown to be effective in a variety of cancers including colon, breast, stomach and pancreatic cancer (42).

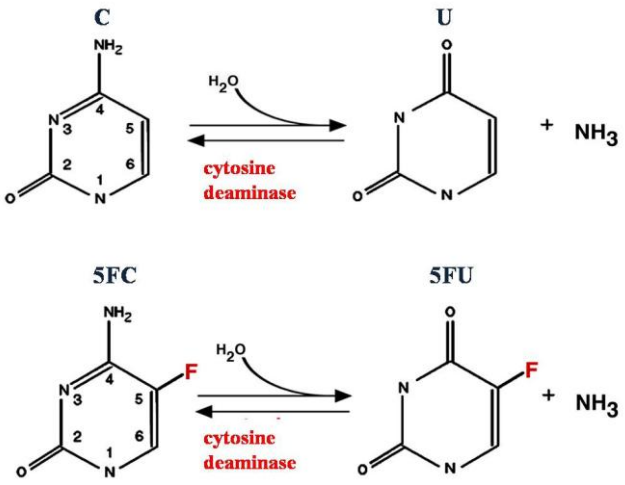


Figure 1.4. Cytosine and 5FC deamination by cytosine deaminase. Cytosine deaminase catalyzes the hydrolytic deamination of cytosine and 5FC to uracil and 5FU, respectively, releasing ammonia (NH_3).

In cells expressing CD, 5FU is converted into several active metabolites: 5-fluoro-2'-deoxyuridine 5'-monophosphate (5FdUMP), 5-fluorodeoxyuridine-triphosphate (5FdUTP) and 5-fluorouridine-triphosphate (5FUTP) (Figure 1.5). Each of these metabolites can cause cell death through different pathways. The first and major mechanism of action of 5FU is by irreversible inhibition of thymidylate synthase (TS) through the formation of a ternary complex between 5FdUMP, TS and 5,10-methylenetetrahydrofolate (CH_2THF). TS is responsible for catalyzing the conversion of

deoxyuridine-monophosphate (dUMP) into deoxythymidine-monophosphate (dTMP) by a reductive methylation reaction with CH_2THF as the methyl donor. Inhibition of TS leads to the depletion of dTMP causing a subsequent decrease in deoxythymidine-triphosphate (dTTP) levels, an essential building block for DNA synthesis, leading to DNA chain termination. The second mechanism of action of 5FU is the false incorporation of 5FdUTP during DNA chain elongation. In the best scenario, this misincorporation is repaired by the enzyme uracil-N-glycosylase (UNG) during nucleotide excision repair. However, this process generally causes further false-nucleotide incorporations, resulting in DNA breaks and cell death (42). Finally, the third 5FU metabolite 5FUTP may also be incorporated into RNA during transcription leading to interference of RNA processing and protein synthesis (43).

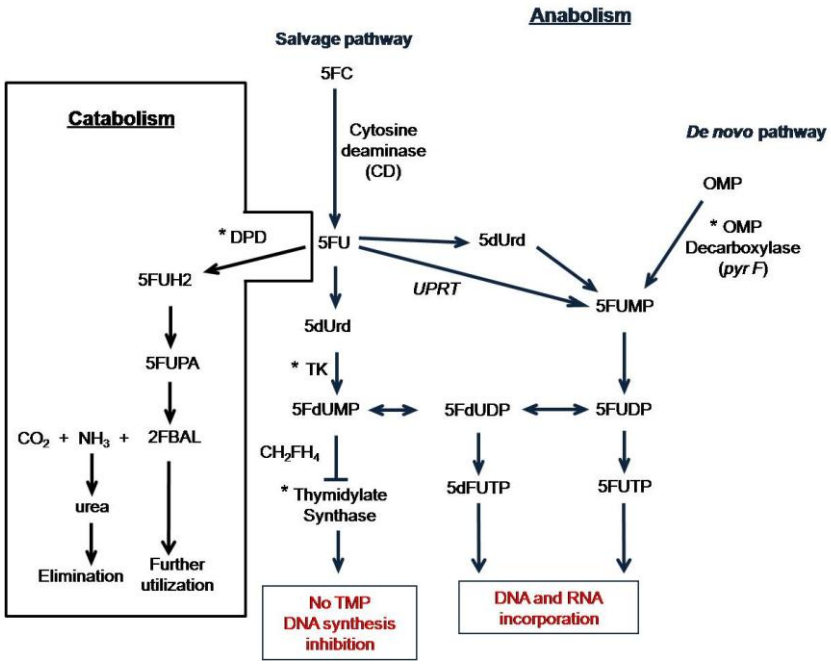


Figure 1.5. 5FC metabolites and their fates in the pyrimidine metabolism pathway. DPD: dihydropyrimidine dehydrogenase, TK: thymidine kinase, UPRT: uracil phosphoribosyltransferase, OMP: orotidine-5'-monophosphate, CH_2FH_4: *N*5,*N*10-methylenetetrahydrofolate, 5FUH2: 5,6-dihydro-5-fluorouracil, 5FUPA: 5-α-fluoro-β-ureidopropionic acid, 2FBAL: 2-α-fluoro-β-alanine, *: endogenous enzymes.

An advantage of CD/5FC over HSV-TK/GCV is the small and uncharged nature of 5FU making it capable to freely diffuse from cell to cell (44). As 5FC is transferred to neighboring non-transfected cells by non-facilitated diffusion, only about 5% of tumor cells expressing cytosine deaminase (CD) were necessary to obtain significant antitumor effect, and direct cell contact was not required (8). The ability to diffuse may be an important factor as cell to cell communication may be diminished in cancer cells as a result of decreased gap junction formation (45, 46). 5FU has also been shown to be a strong clinical radio-sensitizer effective in the treatment of solid tumors (47, 48). Because 5FU asserts its cytotoxic effects in three different ways, it is more difficult if not impossible for cells to repair DNA damage induced by 5FU metabolites which causes cancer cells to display greater sensitivity to radiotherapy. There are two types of CD enzymes currently used in suicide gene therapy: bacterial and yeast, isolated from *Escherichia coli* and *Saccharomyces cerevisiae*, respectively. Although they both deaminate cytosine and 5FC, these enzymes differ from each other in size, catalytic metal, tertiary structure, thermostability and their efficiency in converting 5FC to 5FU (Table 1.1) (16, 49-51). Yeast CD (yCD) has shown superiority in catalytic activity over bacterial CD (bCD) with K_m and V_{max} values 22- and 4-fold lower for 5FC than bCD (49, 52). In comparison studies, yCD has improved radiosensitization, and the bystander effect in cancer cells treated with 5FC is greater than bacterial CD (bCD), presumably due to the difference in catalytic efficiencies (49, 52). Human colon cancer cells (HT29) expressing yCD and irradiated with 1-2 Gy showed a significantly greater radiosensitization over cells expressing bCD, requiring approximately 3-fold lower concentration of 5FC to achieve similar response. Increased radiosensitization by 5FC

in HT29/yCD was determined to be due to an increased production of 5FU rather than a greater sensitivity to either radiation or 5FU (49, 52). *In vivo* bystander experiments using nude mice injected with cells expressing either bCD or yCD, showed only 10% of cells expressing yCD were required to inhibit tumor growth with combinations of 5FC (500 mg/kg) and radiation (3 Gy). The same conditions did not show any bystander effect in mice bearing bCD-expressing tumors which further demonstrate yCD superiority (49, 52). However, when stored at 37°C, yCD is thermolabile with a half life of less than six hours compared to bCD (>100 hours), making yCD less suitable for application in humans (49).

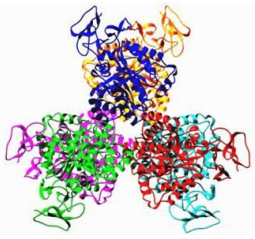
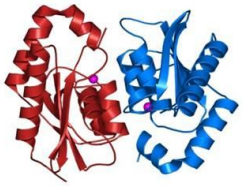
	E. coli	*S. cerevisiae*
Monomer size	48 kDa	17.5 kDa
Catalytic metal	Iron	Zinc
Half life @ 37°C	>100 hrs	< 6 hrs
Kinetic Parameters		
cytosine		
K_m	2.2 mM	3.9 mM
V_{max}	52 (μM/min/μg)	184(μM/min/μg)
5FC		
K_m	18 mM	0.8 mM
V_{max}	12 (μM/min/μg)	68 (μM/min/μg)
		

Table 1.1. Key differences between the bacterial and yeast forms of cytosine deaminase.

Improving Yeast Cytosine Deaminase

As mentioned above, although yCD has been shown to display more desirable kinetics parameters than bCD, its thermolability limits its efficacy in suicide gene therapy protocols. The half-life of yCD at 37°C is about six hours and its melting temperature (T_m) is 52°C. We sought to optimize yCD by increasing thermostability and 5FC activity. Computational design was used to identify possible amino acid substitutions outside the active site of the enzyme that might thermostabilize yCD without affecting enzymatic activity (53). Three amino acid substitutions (A23L, V108I and I140L) were determined to each thermostabilize yCD by increasing the T_m by 2°C. When introducing two of these substitutions into yCD (A23L and I140L), yCD_{double} was created with a half-life of 21 hours and a T_m of 58°C. When adding the last substitution (V108I) to create yCD_{triple}, a higher thermostabilization was obtained with an improvement in half-life from about four hours to approximately 117 hours and an increase in T_m from 52°C to 62°C as compared to the wild type enzyme. Of importance, the kinetic parameters the thermostabilized yCDs displayed towards either cytosine or 5FC were unaltered. In transfected rat C6 glioma cells both yCD_{double} and yCD_{triple} showed a slight decrease in IC_{50} values of 8 mM and 6 mM, respectively, in comparison with an IC_{50} value of >10 mM in wild type yCD (19). To address the goal of improving 5FC activity, regio-specific random mutagenesis was used to target a sequence within the active site of yCD (19). From these experiments several yCD variants were created and found to confer greater sensitivity to 5FC in *E. coli* in comparison with wild type enzyme expressing cells. One of these mutants, the substitution at residue 92 (yCD_{D92E}) from aspartic acid to glutamic acid is located at the homodimer interface of yCD and unexpectedly resulted in

increased thermostabilization with a T_m of 56°C, without alteration in 5FC activity. As with thermostabilized enzymes, greater sensitivity to 5FC in yCD_{D92E} could be explained by the increase in enzyme half-life, which allows an extended conversion of 5FC to 5FU. *In vitro* cytotoxicity assays in transfected rat C6 glioma cells determined yCD_{D92E} sensitivity to 5FC to be similar to yCD_{double} with an IC_{50} of approximately 8.5 mM. In an *in vivo* xenograft tumor model yCD_{D92E} displayed tumor growth inhibition similar to yCD_{triple}. It was then hypothesized that by combining the D92E substitution with yCD_{triple} a super sensitive variant would be obtained. However, although the combination of yCD_{triple} with D92E resulted in a dramatic T_m increase of 16°C over wild type yCD, over thermostabilization proved to be detrimental to 5FC sensitivity as the combined mutant ($yCD_{triple\text{-}D92E}$) displayed an IC_{50} similar to yCD in rat C6 glioma cells. There are ongoing experiments using regio-specific random mutagenesis to improve the prodrug activity of the thermostabilized yCD that we anticipate will translate to both a lower 5FC concentration needed to achieve effective cell killing and complete *in vivo* tumor ablation.

CHAPTER TWO

Evaluation of Yeast Cytosine Deaminase and HSV-1 Thymidine Kinase Fusions in Double Suicide Gene Therapy

ABSTRACT

Suicide gene therapy is a promising cancer treatment that has the potential to specifically target therapy to tumor cells while sparing normal cells from damage. Despite its advantages, there are limitations in the system including poor delivery of the gene to cancer cells and low enzyme activity towards the prodrug requiring administration of high doses of prodrug. One approach to overcome poor prodrug activation is to create a fusion of the cytosine deaminase (CD) and Herpes Simplex Virus-thymidine kinase (HSV-TK) genes in what is known as double suicide gene therapy (DSGT). The rationale behind DSGT is that the use of two enzyme/prodrug systems will allow for a synergistic cytotoxic effect to occur that will ultimately destroy the tumor with lower and safer prodrug doses. Studies have shown that enhanced tumor growth inhibition and radiosensitization are observed when DSGT is utilized with the bacterial CD/TK fusion due to a synergistic effect to combined 5FC and GCV treatment.

Each of the enzymes used in this study, yeast CD (yCD) and HSV-TK have their limitations. yCD is thermolabile with a melting temperature (T_m) of 52°C and a half-life at 37°C of approximately six hours which precludes its use in human subjects. On the other hand, HSV-TK has low activity towards GCV (K_m 47μM) that requires high concentrations of the prodrug to obtain efficacy. Previous studies in our laboratory have

created yCD and TK mutants (yCD_{double}, yCD_{triple} and SR39, respectively), with either improved thermostabilization or improved activity, by using computational design or regio-specific random mutagenesis. As yCD mutants and SR39 showed improved activity and/or a higher tumor killing efficiency in comparison to wild type enzymes, we sought to evaluate the synergistic effect of mutant fusion enzymes. The results obtained suggest that mutant yCDs in fusion with wild type TK have greater prodrug sensitivity, as determined by IC_{50} values, to either 5FC or GCV than fusions with SR39. In synergistic experiments, yCD/TK showed similar IC_{50} values to yCD_{double}/TK or yCD_{double}/SR39 when treated with 1.5 mM 5FC prior to the addition of 3.0 µM GCV. When comparing rat C6 glioma cells transfected with fusions with cells expressing individual enzymes, mutant fusions showed significant improvement in 5FC sensitivity. However, when treated with GCV and compared with SR39 an increased in IC_{50} value was observed.

Further studies need to be done to elucidate whether there is a structural conformational change in SR39 when it is expressed in a fusion with yCD that would explain the apparent decrease in GCV sensitivity. The fusion of yCD/SR39 and 5FC/GCV treatment has shown promising results and is currently being used in Phase III clinical trials for prostate cancer in combination with radiation. However, our results suggest that fusions with TK could be a better choice over yCD/SR39 due to an apparent higher synergistic effect that might translate to lower prodrug doses currently used.

Introduction

Each wild type enzyme/prodrug system discussed in Chapter 1 has shown positive results in clinical studies but still exhibits some limitations such as low enzyme activity, low transduction efficiency due to current delivery systems and toxicity. An approach being used to eliminate these limitations is through combinations of bCD or yCD with HSV-TK as fusions in suicide gene protocols in what is known as double suicide gene therapy (DSGT). DSGT takes advantage of two metabolic pathways that use two entirely different enzyme/prodrug systems to obtain an effective cancer treatment at the lowest dose possible (Figure 2.1) (54). The rationale behind combining drug treatment protocols includes preventing the emergence of drug resistant cells and reducing toxicity to normal cells by decreasing the dose needed to achieve clinically effective results. Several studies have used a bCD/TK fusion construct followed by treatment with a combination of 5FC and GCV and showed a greater tumor inhibition compared with individual treatments (54-57). The bCD/TK fusion was created in 1997 by Freytag and colleagues to test their hypothesis that a combination treatment of 5FC and GCV will provide a synergistic cytotoxic effect (54). Their study showed that co-administration of 5FC and GCV both radiosensitized tumor cells and resulted in a mild synergistic cytotoxic effect. The synergistic cytotoxicity seen with bCD/TK after treatment with 5FC and GCV was suggested to be caused by a shift in dGTP pools after 5FC treatment (57). The known 5FC mechanism of action includes the inhibition of thymidylate synthase, by the metabolite 5FdUMP, which results in the subsequent reduction of dTTP levels causing DNA synthesis termination (42). It is well known that dTTP induces the reduction of guanosine diphosphate (GDP) to its deoxy form (dGDP)

by allosteric regulation of ribonucleotide reductase which catalyzes the reduction of ribonucleotides to form nucleotides necessary for DNA synthesis and cell survival (58). With 5FdUMP-induced thymidylate synthase inhibition and subsequent dTTP depletion, less GDP is reduced to dGDP and, thus, dGTP levels decrease. Because dGTP competes against GCV-TP for DNA incorporation, its depletion results in an increased amount of CGV-TP being incorporated and a greater tumor growth inhibition (57).

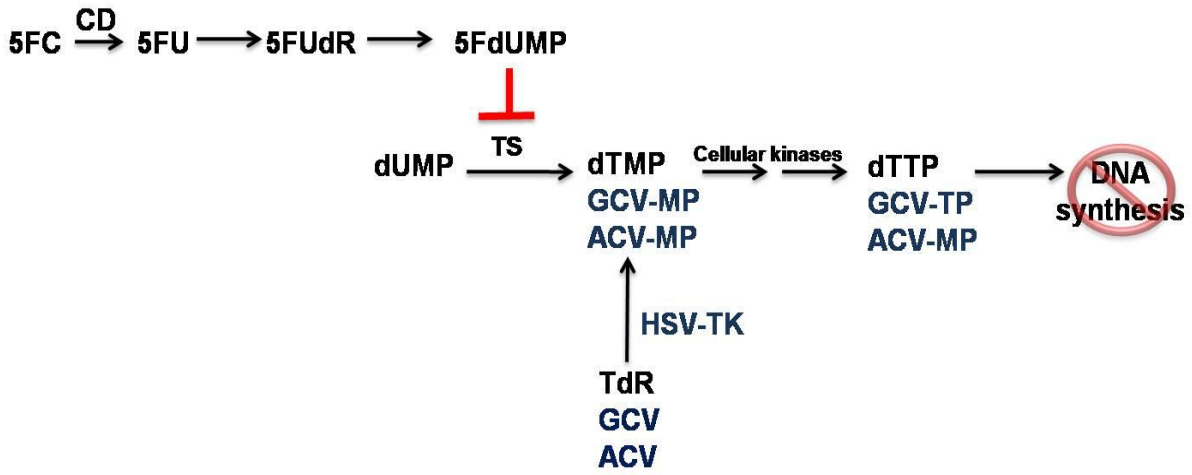


Figure 2.1. CD/5FC and HSV-TK/GCV metabolic pathways (54). CD converts 5FC to 5FU and is further converted by endogenous enzymes to 5FUdR and 5FdUMP which inhibits thymidilate synthase (TS), causing depletion of dTTP and DNA chain termination. HSV-TK converts GCV and ACV into their monophosphorylated form and is further converted by endogenous enzymes to the di- and tri-phosphate form which incorporates into DNA causing cell death.

Some studies using a fusion gene of yCD/SR39 have shown, as with bCD/TK, a greater killing effect and radiosensitization than treatment with single constructs (59, 60). yCD/SR39 is currently being studied in a Phase III clinical trial for prostate cancer where it is delivered by an oncolytic adenovirus followed by 5FC, GCV and radiation treatment. The fusion yCD/SR39 has also been used in a replication-competent adenovirus-mediated suicide gene therapy approach with or without radiation (59, 60).

These studies used prostate and pancreatic tumor models and showed that yCD/SR39 fusion co-expressed with adenovirus death protein (APD) enhanced tumor cell killing without adding toxicity. Expression of ADP makes the vector more cytolytic, allowing for easier tumor cell infection and a facilitated spread to neighboring cells with the possibility of increasing the bystander effect. Results obtained in both studies showed a lack of toxicity and significant tumor control (59, 60). In 2003, a human sodium iodide symporter (hNIS) gene was used in conjunction with yCD/SR39 adenovirus-mediated suicide gene therapy to improve visualization of gene-therapy vectors (61). The hNIS gene was used to transport anions including pertechnetate ($^{99m}TcO_4^-$) which has physical characteristics that could be exploited for nuclear imaging. From that study, a valuable tool for gene therapy clinical trials was created by the utilization of three reporter genes (hNIS, CD and HSV-1 TK), that resulted in a greater imaging resolution allowing for noninvasive visualization of gene expression and vector delivery (61).

Unlike with the fusion of bCD/TK, basic synergistic experiments are yet to be done with yCD/TK or mutant fusions. Because yCD has been shown to more efficiently convert 5FC to 5FU than bCD, one might anticipate that the fusion of yCD/TK will display a more powerful synergistic effect that that observed with bCD/TK. A greater drug synergism with the combination treatment of 5FC and GCV in yCD/TK-expressing cells might therefore translate to a more efficient cancer therapy using low drug doses thereby decreasing side effects while attaining complete tumor ablation. In this study we sought to perform cytotoxicity assays to determine synergistic effects when rat C6 glioma cells express thermostabilized yCD (yCD_{double} and yCD_{triple}) and SR39 as fusion genes. We hypothesized that, by using these mutants in double suicide gene therapy,

we will obtain a dramatic synergistic effect following combination treatment of 5FC and GCV. However, results obtained were unexpected as fusions of yCD or thermostabilized yCD with TK fusions appears to be more sensitive to either 5FC or GCV than fusions with SR39. Our study suggests the use of thermostabilized yCD/TK fusions, instead of yCD/SR39, in ongoing clinical trials might be the most appropriate approach to obtain complete tumor ablation with safer prodrug doses. When comparing our fusions with SR39 alone, a loss in GCV sensitivity was obtained that might be caused by the enzyme impediment to bind GCV due to a partial blockade of the binding site by yCD. Further biochemical and/or structural studies are needed to help us explain what might be happening with fusions of yCD or thermostabilized yCD and SR39 at a molecular level.

Materials and methods

Materials

Oligonucleotides used to introduce mutations and sequence pUB:yCD/TK fusions were obtained from Integrated DNA Technologies (Coralville, IA). Introduction of mutations was done using the QuikChange® II Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA). DNA purification was done using several kits: Wizard® PCR prep kits from Promega (Madison, WI), HiSpeed® Plasmid Mini Kit from Qiagen (Valencia, CA), and StrataPrep® EF Plasmid Midikit from Stratagene (La Jolla, CA). AlamarBlue® was purchased from Serotec Limited (Oxford, UK). All cell culture reagents were purchased from Gibco (Carlsbad, CA). All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Bacterial strains

Escherichia coli strain NM522 [F^+ *lac*I^qΔ(*lac*Z)-M15*pro*A^+B^+/*sup*E *thi*Δ(*lacpro*AB) Δ(*hsd*MS-*mcr*B)5($r_k^-m_k^-$McrBC$^-$)] and *E. coli* strain XL1-Blue [F'::Tn10 *pro*A^+B^+ *lac*I^q D(*lac*Z) *M15/recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* ($r_k^-m_k^+$) *supE44 relA1 lac*] were used as a recipient for certain cloning procedures. *E. coli* strain JM103 [*end*A1 *gln*V44 *sbc*BC *rps*L *thi*-1 Δ(*lac-pro*AB) F'(*traD*36 *pro*AB^+ *lac*I^q *lac*ZΔM15)] was used to obtain plasmid DNA suitable for transfections.

Cell lines

Cell lines were maintained in a humidified incubator at 37 °C in 5% CO_2. Rat C6 glioma cells (C6) were purchased from ATCC (Manasass, VA) and were grown in Dulbecco's Modified Essential Medium (pH 7.2) containing 5% fetal bovine serum, 1 μM sodium pyruvate, 10 mM HEPES, 100 μM nonessential amino acids, 100 U/mL

penicillin G, 10 µg/mL streptomycin sulfate, 292 µg/mL L-glutamine. Transfected cells were cultured in media supplemented with the antibiotic blasticidin at a concentration of 4 µg/mL for selection of stable transfectants.

Introduction of mutations to yCD/TK

The QuikChange® kit was used to introduce amino acid substitutions into pUB:yCD/TK to obtain combinations of wild type yCD, TK and mutant fusion constructs. Refer to Table 2.1 for oligonucleotide information. Substitutions were confirmed by sequencing DNA in the Washington State University Sequencing Core Laboratory using oligo MB400 (5' TCAGTG TTAGACTAGTAAATTGTC3') to sequence the yCD portion and MB468 (5' GCACCGTAT TGGC 3') for the TK portion.

<u>In vitro</u> cytotoxicity assays

In vitro studies were done using the mammalian expression vector pUB (Invitrogen, CA) expressing wild type yCD, TK, fusion constructs and mutants (pUB:yCD$_{double}$, pUB:yCD$_{triple}$, pUB:SR39, pUB:yCD/TK, pUB:yCD/SR39, pUB:yCD$_{double}$/TK or pUB:yCD$_{double}$/SR39, pUB:yCD$_{triple}$/TK or pUB:yCD$_{triple}$/SR39). Transfection in rat C6 glioma cells were done by seeding 150,000 cells into 6-well plates with overnight incubation (37°C, 5% CO_2) followed by the addition of a transfection mix in 1X Dulbecco's modified Eagle's medium (DMEM, pH 7.2) containing 3 µL of the transfection reagent FuGENE® 6 (Roche, Penzberg, Germany) per 1µg of endonuclease-free DNA (ratio 3:1). Stable transfectants were created through multiple passages in media containing the antibiotic blasticidin (1X DMEM, 5% fetal bovine serum (FBS), 100 U/mL penicillin, 10 µg/mL streptomycin, 292 µg/mL glutamine, 10 mM HEPES, 100 µM non-essential amino acids, 1 µM sodium pyruvate, 4 µg/mL blasticidin,

pH 7.2). To obtain IC_{50} values for each fusion construct, cytotoxicity assays were performed where 70% confluent cells were treated with phosphate buffered saline (PBS) (170 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 and 1.7 mM KH_2PO_4), detached with PBS containing 5 mM EDTA and trypsin and seeded into 96-well plates at 500 cells/well. Following overnight attachment, cells were treated with different concentrations of 5FC (0-15 mM) or GCV (0-50 µM) for seven days, at which time the redox indicator dye Alamar blue was added. Cell survival was determined using a multi-detection microplate reader Synergy™ HT and microplate data collection and analysis software Gen5 at a fluorescence wavelength of 530/590nm. Results were plotted as percentage of cell survival versus drug concentration to obtain IC_{50} values for each construct. At least two replicates were performed.

Synergistic experiments

After IC_{50} value determination, a combined treatment of 5FC and GCV was done as in previous studies, where better results were shown when 5FC was administered prior to GCV treatment as opposed to GCV first followed by 5FC (57). Briefly, for the synergistic experiments, transfected cells were seeded at 250 cells/well and treated with 1.5 mM 5FC 24 hr prior to the addition of new media containing 3.0 µM GCV followed by a six-day incubation period. To test if cell survival depended on when cells were treated with GCV after 5FC addition, 5FC treatment was done at 0, 24, 48 and 72 hr. Cell survival was determined as above.

Results and discussion

In vitro cytotoxicity assay experiments

As mentioned in Chapter 1, the use of wild type yeast cytosine deaminase (yCD) and Herpes Simplex Virus-thymidine kinase (HSV-TK) is limited in suicide gene therapy protocols due to low enzyme thermostability and/or poor enzyme activity, requiring high doses of prodrug to achieve an effect in tumor cells. Various mutagenesis approaches have been used to create super mutants as a means to overcome these limitations. The determination of amino acid substitutions in yCD, that thermostabilized the enzyme, allowed for an increased in half-life and melting temperature making yCD more appropriate for clinical use in human subjects (53). Thermostabilization of yCD allowed for an increased in half-life which then provides an extended conversion of 5-fluorocytosine (5FC) into 5-fluorouracil (5FU), thereby resulting in a more effective cell killing. Enzyme kinetic analysis reveals that increased 5FC sensitivity is not due to alteration in kinetic parameters. In terms of HSV-TK, the improvement is due to a greater activity towards ganciclovir (GCV). The HSV-TK mutant, SR39, displays more than 2000-fold reduction in IC_{50} when compared to wild type TK and has proven to be exceptional both *in vitro* and *in vivo* (18, 35, 62). Because of the significant improvement in yCD we questioned whether the fusion of yCD/SR39 currently used in clinical trials is the best candidate for double suicide gene therapy. As there are no published synergistic data for yCD/TK fusions, we hypothesized that by using thermostabilized yCD (yCD_{double} and yCD_{triple}) in fusion with SR39 a stronger synergistic effect would occur in comparison with yCD/TK or yCD/SR39. To test this hypothesis we

sought to first determine the IC_{50} values for 5FC and GCV in cells expressing fusion enzymes to obtain optimal treatment conditions for synergistic experiments.

In vitro studies were done using the mammalian expression vector pUB containing wild type yCD, TK, fusion constructs and mutants (pUB:yCD_{double}, pUB:yCD_{triple}, pUB:SR39, pUB:yCD/TK, pUB:yCD/SR39, pUB:yCD_{double}/TK or pUB:yCD_{double}/SR39, pUB:yCD_{triple}/TK or pUB:yCD_{triple}/SR39). Stably transfected rat C6 glioma cells were seeded in 96-well plates and treated with either 5FC or GCV after overnight incubation. Seven days later Alamar blue was added to each well and fluorescence analysis was done to ascertain cell survival. Percent cell survival was calculated by dividing average fluorescence of treated cells over that in untreated wells for each drug concentration. The obtained values were then plotted against drug concentration and IC_{50} values determined for each construct (Table 2.2). Statistical significance of each fusion construct was evaluated using one way ANOVA and Holm's multiple comparisons (Tables 2.3 and 2.4). Figures 2.2 and 2.3 show representative survival curves of stably transfected cells treated with 5FC or GCV, respectively expressing (A) yCD, (B) yCD_{double} or (C) yCD_{triple} in fusion with either TK or SR39.

Values seen in Table 2.2 are the average IC_{50} of two separate experiments with standard error of the mean. Cells expressing yCD/TK proved to be more sensitive to 5FC with an IC_{50} value of 1.85±0.15 mM compared with 5.55±0.35 mM with yCD/SR39 transfected cells. The same pattern was observed where stably transfected cells were treated with different concentrations of GCV. Once again, yCD/TK showed approximately 6-fold greater sensitivity to GCV than yCD/SR39 expressing cells with IC_{50} values of 1.20±0.10 µM versus 10.15±1.65 µM. The greater sensitivity yCD/TK

displays for either 5FC or GCV individually suggests that the fusion of yCD/SR39 currently used in clinical trials is not the best candidate. Even though fusions of yCD/TK showed greater sensitivity with either drug, as compared with yCD/SR39, the same was not seen with thermostabilized yCD in fusions with TK or SR39. When cells expressing fusion enzymes were treated with 5FC, yCD_{double}/TK displayed a similar IC_{50} value to that of yCD_{double}/SR39 with an IC_{50} values of 0.95±0.55 mM and 2.15±1.35 mM, respectively. In GCV cytotoxicity assays, the fusion of yCD_{double}/TK also demonstrated similar cell sensitivity with an IC_{50} of 0.75±0.25 µM as compared to yCD_{double}/SR39 which displayed a GCV IC_{50} of 1.40±0.00 µM. Triple thermostabilized yCD (yCD_{triple}) in fusion with TK or SR39 showed similar results as yCD_{double} fusions. When C6 cells were treated with 5FC yCD_{triple}/TK displayed an IC_{50} of 0.45±0.25 mM 5FC which proved to be similar to cells expressing yCD_{triple}/SR39 (IC_{50} of 2.25±1.20 mM 5FC). The same was true for C6 cells expressing yCD_{triple}/TK treated with GCV where an IC_{50}-value of 4.85±1.75 µM was similar to that obtained for yCD_{triple}/SR39-expressing cells (2.35±0.45 µM),.

When comparing all fusion constructs tested, fusion of yCD_{triple}/TK conferred a slightly greater 5FC sensitivity with an IC_{50} value of approximately 0.45±0.25 mM. As TK does not contain 5FC activity, lower IC_{50} values observed for yCD in a fusion might be due to yCD stabilization and/or an altered 5FC activity. Because pUB transfected cells displayed an apparent toxicity to 5FC treatment similar to yCD expressing cells, some concerns are raised. Other controls for this study include yCD, yCD_{double} and yCD_{triple} for which IC_{50} values have been previously determined to be over 10 mM, 8 mM and 6 mM, respectively when expressed with the mammalian expression vector pcDNA

(19). However, in the results obtained the values were about 13.45±1.55 mM for yCD, 8.00±1.40 mM for yCD_{double} and 11.05±1.95 mM for yCD_{triple}. Even though the mammalian expression vectors used in each study have different promoters (ubiquitin C (pUB) versus CMV (pcDNA)), the relative degree of 5FC sensitivity for each yCD wild type and mutants should be the same.

As in cytotoxicity assays with 5FC, similar results where obtained when treating cells with GCV in terms of fusions with TK showing similar sensitivity to fusions with SR39, with the exception of yCD/TK which displayed about 6-fold lower IC_{50} for GCV as compared with yCD/SR39. These results were unexpected as it was hypothesized that fusions with SR39 will show greater sensitivity to GCV due to the significant improvement in SR39 activity over TK (29, 32). When comparing each fusion with individual enzymes, yCD_{triple}/TK showed the greatest sensitivity to 5FC only with an 11- to 19-fold decrease in IC_{50} when compared with wild type or mutant yCD alone. In experiments where cells expressing enzymes were treated with GCV only, yCD_{double}/TK displayed a more powerful cytotoxic effect with a 50-fold decrease in IC_{50} when compared to wild type TK. However, none of our SR39 fusions improved cell killing when compared with the GCV IC_{50} obtained for SR39 alone (0.45±0.05 μM). Results observed in fusions expressing SR39 might be explained by the possibility that having yCD in fusion with SR39 might be detrimental for the mutant activity towards GCV. This effect might be due to destabilization or conformational changes in the structure of mutant TK that alters its activity. Results presented in this study are preliminary and need to be confirmed by additional experiments, including western blots to determine protein expression of our fusion constructs and kinetic analysis.

Synergistic experiments

By definition drug synergism is the result of two drugs working in combination to produce an effect greater than the sum of their individual effects (63-65). It has been shown with bCD/TK, that there is mild synergism when cancer cells expressing the fusion were treated with 5FC, GCV and radiation (54, 57). A shift in dGTP pools by 5FC was suggested to be the cause for the synergistic cytotoxicity seen with the greatest impact when GCV is added 24 hours after 5FC treatment, as opposed to simultaneously (57). The 5FC mechanism of action includes inhibition of thymidylate synthase by the metabolite 5FdUMP that results in the subsequent reduction of dTTP levels causing DNA synthesis termination (42). It is well known dTTP induces the reduction of GDP to form deoxyguanosine diphosphate (dGDP) by allosteric regulation of ribonucleotide reductase which is the enzyme responsible for the conversion of ribonucleotides to nucleotides necessary for cell survival (58). Following dTTP depletion, the conversion of dGDP from GDP by ribonuclotide reductase is reduced and, thus, dGTP levels decrease. As dGTP competes with GCV-TP for DNA incorporation, depletion of dGTP pools results in increased amount of CGV-TP being incorporated into DNA causing DNA synthesis inhibition and cell death (57).

Results obtained from *in vitro* cytotoxicity assays were used to study whether there is synergism with the fusion constructs studied here and to determine which one is the best in terms of IC_{50} value and cell killing efficiency. The treatment included either 5FC or GCV as controls or 5FC followed by incubation at different time points prior to GCV treatment. After overnight incubation, cells were treated with either drug or media containing no drug and incubated for seven days. A total of 40 replicates per treatment

were averaged and plotted as percent cell survival of treated cells over non-treated cells. To find the optimal conditions for the combination treatment, cell killing efficiency was tested using different times between treatment with 5FC and treatment with GCV (0, 24, 48 and 72 hr) (data not shown). In accordance to similar experiments in bCD/TK fusions, a greater cell killing was observed when using a 24-hour incubation period between each drug treatment as opposed to treatment with 5FC and GCV simultaneously (57). In their study, Boucher and colleagues also showed better results when treating DU145 prostate carcinoma cells expressing bCD/TK with 5FC followed with GCV, as opposed to treating first with GCV and then 5FC (57). Our results indicate there was no difference in cell survival between a 5FC incubation period of 24 hr and 48 or 72 hours prior to GCV treatment (data not shown).

Following the determination of suitable conditions (time and drug dosing) for treatment, experiments were repeated to determine which fusion construct was exceptional in terms of a greater synergistic effect and an increased cell killing efficiency. Pools of stable transfectants containing empty vector, wild type yCD, TK, fusion constructs and mutants (pUB:yCD$_{double}$, pUB:yCD$_{triple}$, pUB:SR39, pUB:yCD/TK, pUB:yCD/SR39, pUB:yCD$_{double}$/TK or pUB:yCD$_{double}$/SR39, pUB:yCD$_{triple}$/TK or pUB:yCD$_{triple}$/SR39) were seeded at 250 cells/well and treated with either 1.5 mM 5FC (black columns), 3.0 µM GCV(white columns) or a combination of both (crimson columns) (Figure 2.4). In combination treatments GCV was added 24 hr after 5FC treatment. Each column (mean ± SEM; n=1; performed with 40 replicates) is expressed as a percentage of the value for control wells with no treatment. Similar results in cell survival were obtained for C6 cells expressing individual enzymes, with the exception of

TK and SR39 which appears to display some sensitivity to 5FC and GCV combination treatment. As in cytotoxicity assays, cells expressing yCD/TK showed greater sensitivity to combination treatment than yCD/SR39-expressing cells with a cell survival of about 30% versus 70%, respectively. With the thermostabilized double yCD in fusion with TK or SR39 the sensitivity to the 5FC and GCV combination treatment was similar, with a slightly lower cell survival when cells express yCD_{double}/TK. When C6 cells express yCD_{triple} in fusion with TK or SR39, yCD_{triple}/TK displays a greater sensitivity to combination treatment than yCD_{triple}/SR39. With the 5FC and GCV concentrations tested, approximately 50% of cell killing was obtained when cells expressed yCD_{triple}/TK fusion whereas only approximately 34% cell killing was observed in cells expressing yCD_{triple}/SR39 (Table 2.5). For fusions enzymes our results indicate a similar cell killing effect (about 67%) for cells expressing yCD/TK, yCD_{double}/TK and yCD_{double}/SR39. These results were in accordance with what was obtained in cytotoxicity assays (Figure 2.2 and 2.3) where cells expressing yCD/TK, yCD_{double}/TK and yCD_{double}/SR39 showed slightly greater sensitivity with individual treatments. We plan to repeat synergistic experiments with lower concentrations of each drug, for example 0.5 mM 5FC and 1.5 µM GCV, to be able to discriminate between fusion constructs.

In drug synergy the therapeutic or toxic effect seen when two or more compounds are used together is greater than the sum of each individual drug. In accordance to Figure 2.4 and Table 2.5, it could be concluded that no synergy, but an additive effect, was observed with all fusion constructs tested. The preliminary results obtained, even though unexpected, gave us more insight of the effect present when using combination of enzyme/prodrug systems. DSGT has great potential to improve

current suicide gene therapy outcomes with the possibility to reduce drug doses used in clinical trials that could lead to a decreased or absence of side effects. The development of tumors resistant to treatment can also be reduced or abolished by combination of enzyme/prodrug/radiation therapy.

FIGURE LEGENDS

Figure 2.2. 5FC sensitivity assays of rat C6 transfectants.

Pools of stable transfectants containing **(A)** vector only pUB (●), pUB:yCD (□), pUB:TK (◆), pUB:SR39 (◇) and yCD wild type, or **(B)** yCD_{double}, or **(C)** yCD_{triple} in fusion with TK (▲) or SR39 (△) were evaluated for 5FC sensitivity as described in the Materials and Methods section. After seven days of 5FC treatment, cell survival was determined using the detection system Alamar blue according to the manufacturer's instructions. Each data point (mean ± SEM; n=2; performed with 24 replicates) is expressed as a percentage of the value for control wells with no 5FC treatment. The experiment was repeated twice with similar results.

Figure 2.3. GCV sensitivity assays of rat C6 transfectants.

Pools of stable transfectants containing **(A)** vector only pUB (●), pUB:yCD (□), pUB:TK (◆), pUB:SR39 (◇) and yCD wild type, or **(B)** yCD_{double}, or **(C)** yCD_{triple} in fusion with TK (▲) or SR39 (△) were evaluated for GCV sensitivity as described in the Materials and Methods section. After seven days of GCV treatment, cell survival was determined using the detection system Alamar Blue according to the manufacturer's instructions. Each data point (mean ± SEM; n=2, performed with 24 replicates) is expressed as a percentage of the value for control wells with no GCV treatment. The experiment was repeated twice with similar results.

Figure 2.4. Synergistic experiment with 5FC and GCV combination treatment

Pools of stable transfectants containing empty vector, wild type yCD, TK, fusion constructs and mutants (pUB:yCD$_{double}$, pUB:yCD$_{triple}$, pUB:SR39, pUB:yCD/TK, pUB:yCD/SR39, pUB:yCD$_{double}$/TK or pUB:yCD$_{double}$/SR39, pUB:yCD$_{triple}$/TK or pUB:yCD$_{triple}$/SR39) were seeded at 250 cells/well and treated with either 1.5 mM 5FC (black columns), 3.0 µM GCV(white columns) or a combination (crimson columns). In combination treatment GCV was added 24 hr after 5FC treatment. Each column (mean ± SEM; n=1; performed with 40 replicates) is expressed as a percentage of the value for control wells with no treatment.

Table 2.1. Oligonucleotides used to introduce SR39, yCD_{double} or yCD_{triple} into yCD/TK fusion constructs. Letters in **bold** indicate the base substitutions to obtain each variant.

Oligonucleotides for SR39	
MB449	5' GCCCTCACC**A**TC**T**TC**C**TCGACCGCCATCCC 3'
MB450	5' GGGATGGCGGTCGA**G**GA**A**GA**T**GGTGAGGGC 3'
MB451	5' CCATCCCATCGCC**TT**C**A**T**G**CTGTGCTACCCG 3'
MB452	5' CGGGTAGCACAG**C**A**T**G**AA**GGCGATGGGATGG 3'
Oligonucleotides for yCD_{double}	
MB462	5' GCCTATGAGGAGGCG**CT**CTTAGGTTACAAAGAGGG 3'
MB463	5' CCCTCTTTGTAACCTAAG**AG**CGCCTCCTCATAG 3'
MB466	5' GACGATGAGAGGTGTAAAAAG**C**TCATGAAACAATTTATCG 3'
MB467	5' CGATAAATTGTTTCATGA**G**CTTTTTACACCTCTCATCGTC 3'
Oligonucleotides for yCD_{triple}	
MB462-463 MB466-467	Same oligonucleotides as in yCD_{double}
MB464	5' GGTATTCCACGCTGTGTT**A**TCGGTGAGAACG 3'
MB465	5' CGTTCTCACCGA**T**AACACAGCGTGGAATACC 3'

Table 2.2. IC_{50} values of rat C6 glioma cells expressing individual enzymes, wild type fusion or mutant fusion enzymes to 5FC or GCV.

Construct	**IC_{50} for 5FC** (mM)	**IC_{50} for GCV** (μM)
pUB	11.35±0.85	>50.0
pUB:yCD	13.45±1.55	>50.0
pUB:yCD_{double}	8.00±1.40	>50.0
pUB:yCD_{triple}	11.05±1.95	>50.0
pUB:TK	12.95±3.05	>50.0
pUB:SR39	13.3±3.70	0.45±0.05
pUB:yCD/TK	1.85±0.15	1.20±0.10
pUB:yCD/SR39	5.55±0.35	10.15±1.65
pUB:yCD_{double}/TK	0.95±0.55	0.75±0.25
pUB:yCD_{double}/SR39	2.15±1.35	1.40±0.00
pUB:yCD_{triple}/TK	0.45±0.25	4.85±1.75
pUB:yCD_{triple}/SR39	2.20±1.20	2.35±0.45

Table 2.3. Statistically significant differences in IC_{50} values for 5FC treatments.

	pUB:yCD/ TK	pUB:yCD/ SR39	pUB:yCD$_{double}$/ TK	pUB:yCD$_{triple}$/ TK
pUB:yCD	*	*		
pUB:yCD$_{double}$			*	
pUB:yCD$_{triple}$				*
pUB:yCD/TK		*		
pUB:yCD/SR39	*			

*p-value < 0.05
Constructs not shown did not display statistical significance.

Table 2.4. Statistically significant differences in IC_{50} values for GCV treatments.

	pUB:yCD/ TK	pUB:yCD/ SR39	pUB:yCD$_{double}$/ TK	pUB:yCD$_{double}$/ SR39	pUB:yCD$_{triple}$/ TK
pUB:TK	**		**		*
pUB:SR39		*		*	
pUB:yCD/TK		*			
pUB:yCD/SR39	*				

*p-value < 0.05
**p-value < 0.0001
Constructs not shown did not display statistical significance.

Table 2.5. Percent cell killing of rat C6 glioma cells expressing fusion constructs and treated with 1.5 mM 5FC, 3.0 μM GCV or combination treatment.

Construct	% Cell Killing		
	5FC (1.5mM)	GCV (3.0μM)	Combination treatment
pUB:yCD/TK	20.58±2.11	57.54±3.56	66.83±3.37
pUB:yCD/SR39	7.20±0.71	24.68±2.21	32.00±1.61
pUB:yCD$_{double}$/TK	15.22±1.22	48.82±2.74	69.24±3.70
pUB:yCD$_{double}$/SR39	19.70±1.63	51.36±3.03	66.28±3.09
pUB:yCD$_{triple}$/TK	28.68±1.58	20.06±1.40	48.19±2.34
pUB:yCD$_{triple}$/SR39	12.46±1.52	22.23±1.90	33.89±1.55

Figure 2.2

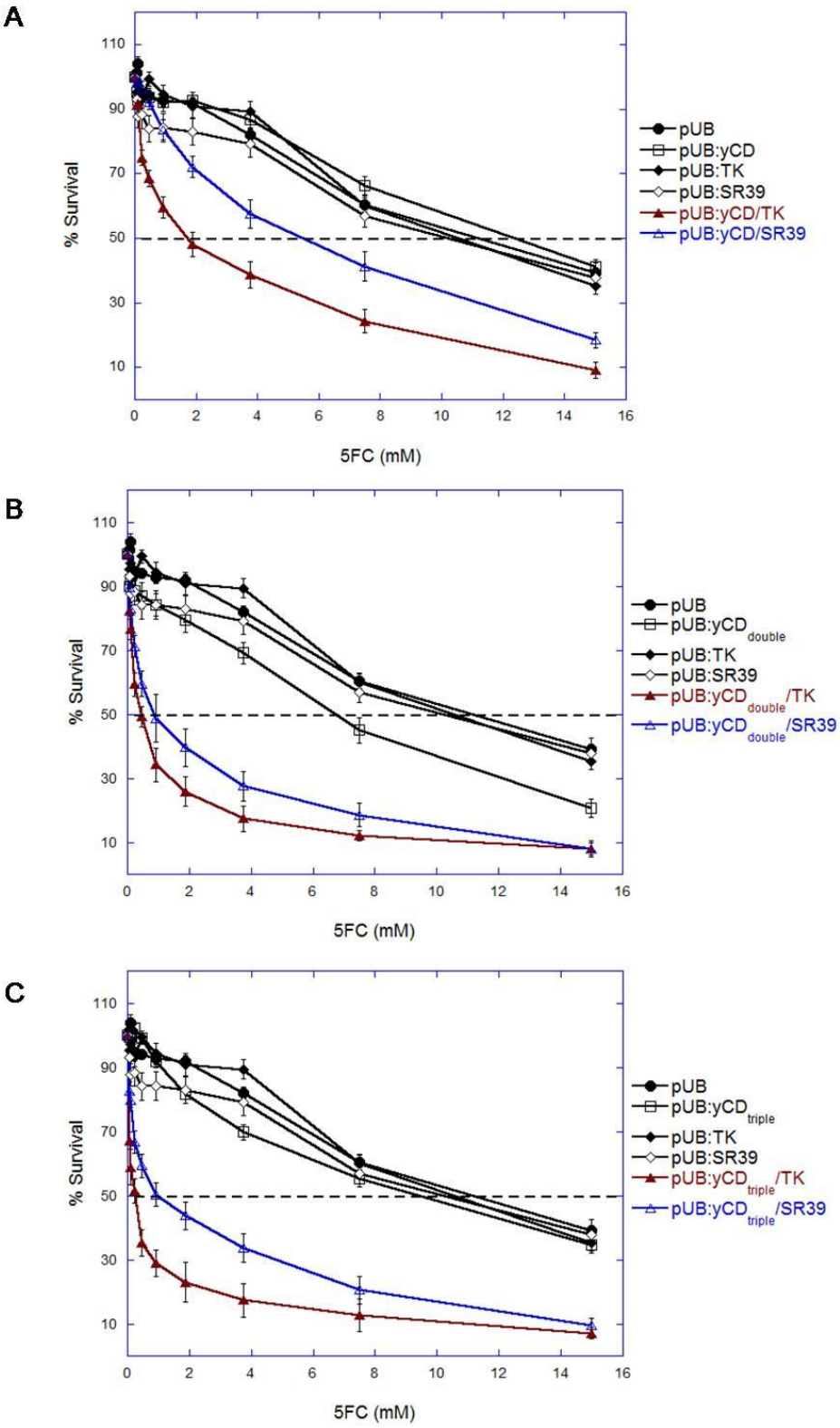

A
% Survival
5FC (mM)
pUB
pUB:yCD
pUB:TK
pUB:SR39
pUB:yCD/TK
pUB:yCD/SR39
B
% Survival
5FC (mM)
pUB
pUB:yCD double
pUB:TK
pUB:SR39
pUB:yCD double /TK
pUB:yCD double /SR39
C
% Survival
5FC (mM)
pUB
pUB:yCD triple
pUB:TK
pUB:SR39
pUB:yCD triple /TK
pUB:yCD triple /SR39

Figure 2.3

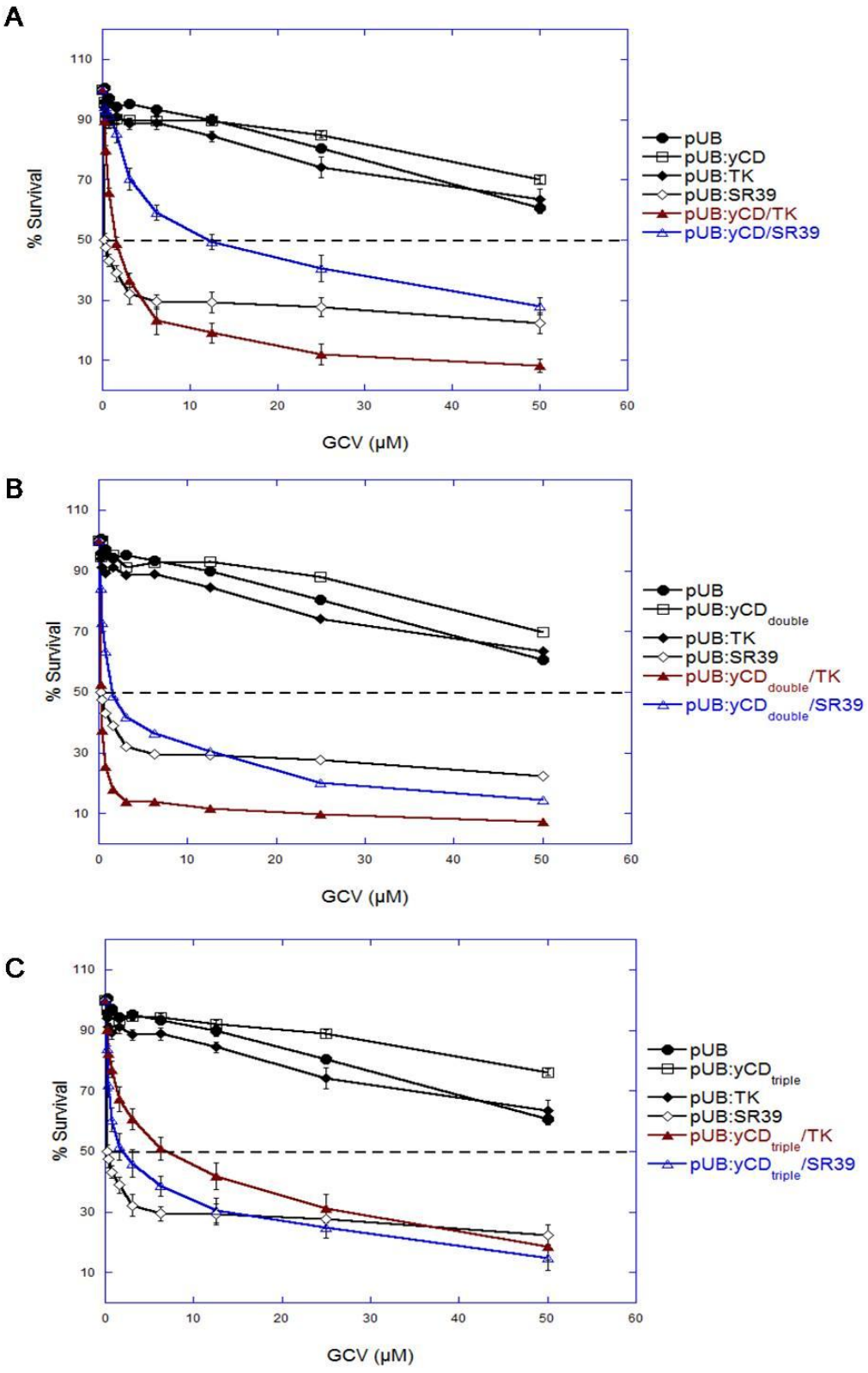
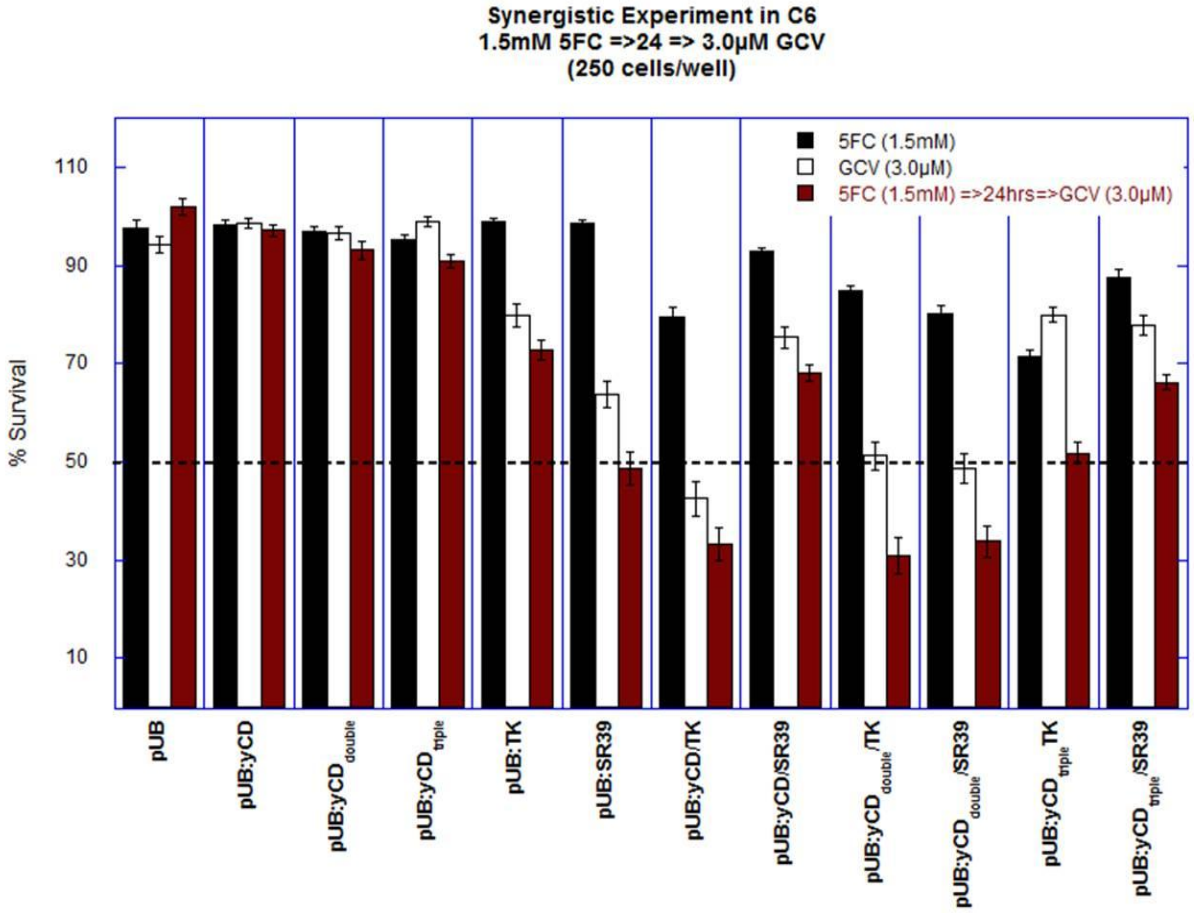

A
% Survival
GCV (μM)
pUB
pUB:yCD
pUB:TK
pUB:SR39
pUB:yCD/TK
pUB:yCD/SR39
B
% Survival
GCV (μM)
pUB
pUB:yCD double
pUB:TK
pUB:SR39
pUB:yCD double /TK
pUB:yCD double /SR39
C
% Survival
GCV (μM)
pUB
pUB:yCD triple
pUB:TK
pUB:SR39
pUB:yCD triple /TK
pUB:yCD triple /SR39

Figure 2.4


Synergistic Experiment in C6
1.5mM 5FC =>24 => 3.0µM GCV
(250 cells/well)
5FC (1.5mM)
GCV (3.0µM)
5FC (1.5mM) =>24hrs=>GCV (3.0µM)
% Survival
110
90
70
50
30
10
pUB
pUB:yCD
pUB:yCD double
pUB:yCD triple
pUB:TK
pUB:SR39
pUB:yCD/TK
pUB:yCD/SR39
pUB:yCD double /TK
pUB:yCD double /SR39
pUB:yCD triple TK
pUB:yCD triple /SR39

CHAPTER THREE

Summary and Future Directions

By definition drug synergism occurs when two or more drugs interact to enhance or magnify the effects of each individual drug. Drug synergism has been studied for decades in an attempt to find more effective drug treatments that will result in less toxic effects and reduced drug resistance (63-65). In suicide gene therapy this is particularly important because of the side effects that chemotherapeutic drugs pose to the cancer patient, such as nausea, vomiting and/or bone marrow depression (3, 66, 67). To this end, a new approach to suicide gene therapy, double suicide gene therapy (DSGT), was created by expressing fusion enzymes in cancer cells followed by treatment with two different drugs. Synergistic effects were observed with bCD/TK fusion in the late 1990s in which cells expressing the fusion and treated simultaneously with 5FC and GCV displayed greater cell killing and radiosensitization of tumor cells when compared to treatment with each individual prodrug (54, 57).

Unlike the bCD/TK system, synergistic experiments have yet to be done with the yCD/TK system despite the advancement of yCD/SR39 to clinical trials. Fusion of yCD/SR39 is currently being evaluated in Phase III clinical trials for prostate cancer in combination with 5FC, GCV and radiation. In this current study we sought to test thermostabilized yCD (yCD_{double} and yCD_{triple}) and SR39 as fusion genes to test the hypothesis that by using these mutants we will obtain a more potent synergistic effect with 5FC and GCV treatment as compared to wild type fusions. A greater synergistic effect for this system was anticipated because it has been shown yCD is superior to bCD in converting 5FC to 5FU (49, 52). In addition, mutants of yCD and HSV-TK

have demonstrated to be superior to wild type enzymes in terms of thermostability or activity towards the prodrug (19, 29, 32, 53).

To test our hypothesis, *in vitro* cytotoxic assays and synergistic experiments were done in rat C6 glioma cells stably transfected with empty vector (pUB), individual enzymes/mutants or fusions constructs and treated with 5FC or GCV individually, or both drugs. Greater synergistic and cell killing effects were expected with fusions of thermostabilized yCD and SR39 because of the significant improvements obtained with each variant in terms of thermostabilization (yCD_{double} and yCD_{triple}) and increased prodrug activity (SR39) (18, 53). However, preliminary results obtained in this study were unexpected as fusions of yCD or thermostabilized yCD with wild type TK appear to be slightly more sensitive to combination treatment than fusions with SR39. As SR39 has been shown to be superior to wild type TK in terms of providing a greater killing effect *in vitro* and *in vivo*, we thought that perhaps the fusion of thermostabilized yCD with SR39 would give a more powerful synergistic effect that could be translated to more potent tumor ablation at low drug doses. When comparing with yCD wild type, the increased sensitivity of fusion constructs treated with 5FC alone may be due to yCD stabilization by having the enzyme in a fusion with TK. Another explanation could be an increased in 5FC sensitivity when yCD is expressed in fusion with TK. Previous studies have created a recombinant fusion protein (LinkCD) with yCD and a linear polysaccharide, hyalorunan, and found that there was a slight increase in T_m of 4°C over wild type yCD (68). The T_m obtained with the yCD mutants, yCD_{double} and yCD_{triple}, were 6°C and 10°C higher, respectively, than that of wild type yCD (53). Although the increase in T_m of LinkCD is lower than that obtained in thermostabilized

yCD_{double} and yCD_{triple}, there was a significant increase in 5FC sensitivity as well as increased survival rate of Balb/c mice bearing C26 murine adenocarcinoma (68).

In vitro cytotoxicity assays for GCV showed that cells expressing yCD_{double}/TK displayed similar sensitivity to GCV than fusions with SR39 (yCD_{double}/SR39) The same result was obtained for yCD_{triple} in fusion with TK or SR39 which also displayed similar IC_{50} values. Even though fusion constructs tested showed approximately 25- to 100-fold lower IC_{50} than wild type TK alone, results obtained are not as promising as when cells express SR39 alone, which have a previously determined IC_{50} for GCV of 0.02 µM (35). Results observed in yCD/SR39 fusions might be explained by the possibility that having yCD in fusion with SR39 might be detrimental for GCV activity. This effect might be due to destabilization or conformational changes in the structure of mutant TK that alter its activity or its ability to bind GCV. As results indicate, a decreased in GCV sensitivity in yCD, yCD_{double} or yCD_{triple} in fusion with SR39 translated to higher IC_{50} values and greater cell survival when compared with SR39-expressing cells.

To elucidate the reason behind the unexpected results obtained in this study, experiments such as enzyme assays and structural studies are necessary. Enzyme assays with thymidine and GCV will help to determine a decrease in activity in the fusion constructs expressing SR39 when compared with SR39 by itself. Structural studies, for example X-ray crystallography, might give us an insight of a possible conformational change in fusions of yCD/SR39 that could further explain our results. One of the mutations contained in SR39 is the substitution of phenylalanine at alanine 168 which was suggested to cause an opening of the active site that allows better

binding of larger substrates such as GCV (62). This raises the question of whether yCD is somehow affecting the side chains displayed by A168F that allows an opening of the active site which then decreases the amount of GCV bound to the enzyme. In that case the opening in the active site of SR39 in fusion to yCD (wild type or mutant) could be similar to that in wild type TK or smaller which could explain why fusions display similar sensitivity to GCV. This might be easily solved by increasing the linker between each enzyme.

In synergistic experiments, fusions of yCD/TK, yCD_{double}/TK and yCD_{double}/SR39 showed similar cytotoxicity to 5FC and GCV combination treatment tested with an approximate cell survival of 30% as compared to individual treatment. Additional experiments are necessary to discriminate between fusion constructs by further lowering the concentrations used of each prodrug in combination treatment. To test this we plan to repeat combination treatment experiments with lower concentrations of each drug, for example 0.5 mM 5FC and 1.5 µM GCV, that will allow us to determine the most exceptional fusion construct in terms of greater cell killing efficiency. The next step after synergistic experiments is to study the bystander effect of cells expressing fusion constructs to determine which fusion is the best in terms of killing non-transfected cells with the least amount of transfected cells present.

In summary, preliminary results from this study opened more questions about the efficacy of yCD thermostabilized enzymes in fusion with TK or SR39 in DSGT. These questions need to be addressed in more extensive studies that could explain our results before moving to an *in vivo* xenograft tumor model. Results suggest that fusions with TK could be a better choice over yCD/SR39 for clinical trials due to an

apparent greater sensitivity to combination treatment that might translate to lower prodrug doses currently used. Synergistic experiments using bCD or yCD in fusion with other TK mutants might also give us another alternative to improve current DSGT outcomes.

The superiority of yCD over bCD in terms of 5FC activity and radiosensitization, the successful thermostabilization of yCD and the creation of super TK mutants, like SR39, make us strongly believe that thermostabilized yCD/TK fusions would be much more successful than bCD/TK fusions in double suicide gene therapy. This could potentially lead to a more powerful cell killing effect thus requiring lower prodrug doses and radiation that might translate to the absence of side effects to patients and a complete tumor ablation. A comparison study between bCD/TK and thermostabilized yCD/TK fusions could be useful to answers all these important questions. Because of the advantages DSGT pose, we believe the determination of fusion enzyme/prodrugs systems as the best candidate for cancer therapy would take us a step closer to curing cancer.

FUNDING

This work was supported by National Institutes of Health grants [CA85939 (M.E.B.) and CA97328 (Barry L. Stoddard and M.E.B.)].

CHAPTER FOUR

References

1. Longley, D. B., and Johnston, P. G. (2005) Molecular mechanisms of drug resistance, *J Pathol 205*, 275-292.

2. Relling, M. V., Rubnitz, J. E., Rivera, G. K., Boyett, J. M., Hancock, M. L., Felix, C. A., Kun, L. E., Walter, A. W., Evans, W. E., and Pui, C. H. (1999) High incidence of secondary brain tumours after radiotherapy and antimetabolites, *Lancet 354*, 34-39.

3. Kaldor, J. M., Day, N. E., Pettersson, F., Clarke, E. A., Pedersen, D., Mehnert, W., Bell, J., Host, H., Prior, P., Karjalainen, S., and et al. (1990) Leukemia following chemotherapy for ovarian cancer, *N Engl J Med 322*, 1-6.

4. Curtis, R. E., Boice, J. D., Jr., Stovall, M., Bernstein, L., Greenberg, R. S., Flannery, J. T., Schwartz, A. G., Weyer, P., Moloney, W. C., and Hoover, R. N. (1992) Risk of leukemia after chemotherapy and radiation treatment for breast cancer, *N Engl J Med 326*, 1745-1751.

5. Travis, L. B., Curtis, R. E., Glimelius, B., Holowaty, E. J., Van Leeuwen, F. E., Lynch, C. F., Hagenbeek, A., Stovall, M., Banks, P. M., Adami, J., and et al. (1995) Bladder and kidney cancer following cyclophosphamide therapy for non-Hodgkin's lymphoma, *J Natl Cancer Inst 87*, 524-530.

6. Moser, E. C., Noordijk, E. M., van Leeuwen, F. E., Baars, J. W., Thomas, J., Carde, P., Meerwaldt, J. H., van Glabbeke, M., and Kluin-Nelemans, H. C. (2006) Risk of second cancer after treatment of aggressive non-Hodgkin's lymphoma; an EORTC cohort study, *Haematologica 91*, 1481-1488.

7. Hatefi, A., and Canine, B. F. (2009) Perspectives in vector development for systemic cancer gene therapy., *Gene Ther Mol Biol 13*, 15-19.

8. Huber, B. E., Austin, E. A., Richards, C. A., Davis, S. T., and Good, S. S. (1994) Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase, *Proc Natl Acad Sci U S A 91*, 8302-8306.

9. Lawrence, T. S., Rehemtulla, A., Ng, E. Y., Wilson, M., Trosko, J. E., and Stetson, P. L. (1998) Preferential cytotoxicity of cells transduced with cytosine deaminase compared to bystander cells after treatment with 5-flucytosine, *Cancer Res 58*, 2588-2593.

10. Elshami, A. A., Saavedra, A., Zhang, H., Kucharczuk, J. C., Spray, D. C., Fishman, G. I., Amin, K. M., Kaiser, L. R., and Albelda, S. M. (1996) Gap junctions play a role in the 'bystander effect' of the herpes simplex virus thymidine kinase/ganciclovir system in vitro, *Gene Ther 3*, 85-92.

11. Mesnil, M., Piccoli, C., Tiraby, G., Willecke, K., and Yamasaki, H. (1996) Bystander killing of cancer cells by herpes simplex virus thymidine kinase gene is mediated by connexins, *Proc Natl Acad Sci U S A 93*, 1831-1835.

12. Mesnil, M., and Yamasaki, H. (2000) Bystander effect in herpes simplex virus-thymidine kinase/ganciclovir cancer gene therapy: role of gap-junctional intercellular communication, *Cancer Res 60*, 3989-3999.

13. Freeman, S. M., Abboud, C. N., Whartenby, K. A., Packman, C. H., Koeplin, D. S., Moolten, F. L., and Abraham, G. N. (1993) The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified, *Cancer Res 53*, 5274-5283.

14. Niculescu-Duvaz, I., and Springer, C. J. (2005) Introduction to the background, principles, and state of the art in suicide gene therapy, *Mol Biotechnol 30*, 71-88.

15. Black, M. E. (2001) Enzyme and pathway engineering for suicide gene therapy, *Genet Eng (N Y) 23*, 113-127.

16. Devanathan, S., Willmon, C., Mahan, S., and Black, M. (2002) Engineering enzymes for improved cancer gene therapy, pp 315-326, Res. Adv. in Cancer.

17. Mahan, S. D., Ireton, G. C., Knoeber, C., Stoddard, B. L., and Black, M. E. (2004) Random mutagenesis and selection of Escherichia coli cytosine deaminase for cancer gene therapy, *Protein Eng Des Sel 17*, 625-633.

18. Black, M. E., Kokoris, M. S., and Sabo, P. (2001) Herpes simplex virus-1 thymidine kinase mutants created by semi-random sequence mutagenesis improve prodrug-mediated tumor cell killing, *Cancer Res 61*, 3022-3026.

19. Stolworthy, T. S., Korkegian, A. M., Willmon, C. L., Ardiani, A., Cundiff, J., Stoddard, B. L., and Black, M. E. (2008) Yeast cytosine deaminase mutants with increased thermostability impart sensitivity to 5-fluorocytosine, *J Mol Biol 377*, 854-869.

20. Kurtz, J. E., and Black, M. E. (2004) Enhancement of suicide gene prodrug activation by random mutagenesis, *Methods Mol Med 90*, 331-344.

21. Moolten, F. L. (1986) Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy, *Cancer Res 46*, 5276-5281.

22. Mullen, C. A., Kilstrup, M., and Blaese, R. M. (1992) Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system, *Proc Natl Acad Sci U S A 89*, 33-37.

23. Singhal, S., and Kaiser, L. R. (1998) Cancer chemotherapy using suicide genes, *Surg Oncol Clin N Am 7*, 505-536.

24. Fillat, C., Carrio, M., Cascante, A., and Sangro, B. (2003) Suicide gene therapy mediated by the Herpes Simplex virus thymidine kinase gene/Ganciclovir system: fifteen years of application, *Curr Gene Ther 3*, 13-26.

25. Bi, W. L., Parysek, L. M., Warnick, R., and Stambrook, P. J. (1993) In vitro evidence that metabolic cooperation is responsible for the bystander effect observed with HSV tk retroviral gene therapy, *Hum Gene Ther 4*, 725-731.

26. Fick, J., Barker, F. G., 2nd, Dazin, P., Westphale, E. M., Beyer, E. C., and Israel, M. A. (1995) The extent of heterocellular communication mediated by gap junctions is predictive of bystander tumor cytotoxicity in vitro, *Proc Natl Acad Sci U S A 92*, 11071-11075.

27. Dilber, M. S., Abedi, M. R., Christensson, B., Bjorkstrand, B., Kidder, G. M., Naus, C. C., Gahrton, G., and Smith, C. I. (1997) Gap junctions promote the bystander effect of herpes simplex virus thymidine kinase in vivo, *Cancer Res 57*, 1523-1528.

28. Greco, O., and Dachs, G. U. (2001) Gene directed enzyme/prodrug therapy of cancer: historical appraisal and future prospectives, *J Cell Physiol 187*, 22-36.

29. Black, M. E., Newcomb, T. G., Wilson, H. M., and Loeb, L. A. (1996) Creation of drug-specific herpes simplex virus type 1 thymidine kinase mutants for gene therapy, *Proc Natl Acad Sci U S A 93*, 3525-3529.

30. Mercer, K. E., Ahn, C. E., Coke, A., Compadre, C. M., and Drake, R. R. (2002) Mutation of herpesvirus thymidine kinase to generate ganciclovir-specific kinases for use in cancer gene therapies, *Protein Eng 15*, 903-911.

31. Degreve, B., Esnouf, R., De Clercq, E., and Balzarini, J. (2000) Selective abolishment of pyrimidine nucleoside kinase activity of herpes simplex virus type 1 thymidine kinase by mutation of alanine-167 to tyrosine, *Mol Pharmacol 58*, 1326-1332.

32. Black, M. E., and Loeb, L. A. (1993) Identification of important residues within the putative nucleoside binding site of HSV-1 thymidine kinase by random sequence selection: analysis of selected mutants in vitro, *Biochemistry 32*, 11618-11626.

33. Pantuck, A. J., Matherly, J., Zisman, A., Nguyen, D., Berger, F., Gambhir, S. S., Black, M. E., Belldegrun, A., and Wu, L. (2002) Optimizing prostate cancer

suicide gene therapy using herpes simplex virus thymidine kinase active site variants, *Hum Gene Ther 13*, 777-789.

34. Wiewrodt, R., Amin, K., Kiefer, M., Jovanovic, V. P., Kapoor, V., Force, S., Chang, M., Lanuti, M., Black, M. E., Kaiser, L. R., and Albelda, S. M. (2003) Adenovirus-mediated gene transfer of enhanced Herpes simplex virus thymidine kinase mutants improves prodrug-mediated tumor cell killing, *Cancer Gene Ther 10*, 353-364.

35. Ardiani, A., Sanchez-Bonilla, M., and Black, M. E. (2009) Fusion enzymes containing HSV-1 thymidine kinase mutants and guanylate kinase enhance prodrug sensitivity in vitro and in vivo, *Cancer Gene Ther*.

36. Gambhir, S. S., Bauer, E., Black, M. E., Liang, Q., Kokoris, M. S., Barrio, J. R., Iyer, M., Namavari, M., Phelps, M. E., and Herschman, H. R. (2000) A mutant herpes simplex virus type 1 thymidine kinase reporter gene shows improved sensitivity for imaging reporter gene expression with positron emission tomography, *Proc Natl Acad Sci U S A 97*, 2785-2790.

37. Pantuck, A. J., Berger, F., Zisman, A., Nguyen, D., Tso, C. L., Matherly, J., Gambhir, S. S., and Belldegrun, A. S. (2002) CL1-SR39: A noninvasive molecular imaging model of prostate cancer suicide gene therapy using positron emission tomography, *J Urol 168*, 1193-1198.

38. Yang, H., Berger, F., Tran, C., Gambhir, S. S., and Sawyers, C. L. (2003) MicroPET imaging of prostate cancer in LNCAP-SR39TK-GFP mouse xenografts, *Prostate 55*, 39-47.

39. Yaghoubi, S. S., Barrio, J. R., Namavari, M., Satyamurthy, N., Phelps, M. E., Herschman, H. R., and Gambhir, S. S. (2005) Imaging progress of herpes simplex virus type 1 thymidine kinase suicide gene therapy in living subjects with positron emission tomography, *Cancer Gene Ther 12*, 329-339.

40. Jacobs, A. H., Rueger, M. A., Winkeler, A., Li, H., Vollmar, S., Waerzeggers, Y., Rueckriem, B., Kummer, C., Dittmar, C., Klein, M., Heneka, M. T., Herrlinger, U., Fraefel, C., Graf, R., Wienhard, K., and Heiss, W. D. (2007) Imaging-guided gene therapy of experimental gliomas, *Cancer Res 67*, 1706-1715.

41. Likar, Y., Dobrenkov, K., Olszewska, M., Shenker, L., Cai, S., Hricak, H., and Ponomarev, V. (2009) PET imaging of HSV1-tk mutants with acquired specificity toward pyrimidine- and acycloguanosine-based radiotracers., *Eur J Nucl Med Mol Imaging 36*, 1273-1282.

42. Longley, D. B., Harkin, D. P., and Johnston, P. G. (2003) 5-fluorouracil: mechanisms of action and clinical strategies, *Nat Rev Cancer 3*, 330-338.

43. Ghoshal, K., and Jacob, S. T. (1994) Specific inhibition of pre-ribosomal RNA processing in extracts from the lymphosarcoma cells treated with 5-fluorouracil, *Cancer Res 54*, 632-636.

44. Domin, B. A., Mahony, W. B., and Zimmerman, T. P. (1993) Transport of 5-fluorouracil and uracil into human erythrocytes, *Biochem Pharmacol 46*, 503-510.

45. Mesnil, M., Crespin, S., Avanzo, J. L., and Zaidan-Dagli, M. L. (2005) Defective gap junctional intercellular communication in the carcinogenic process, *Biochim Biophys Acta 1719*, 125-145.

46. Wilgenbus, K. K., Kirkpatrick, C. J., Knuechel, R., Willecke, K., and Traub, O. (1992) Expression of Cx26, Cx32 and Cx43 gap junction proteins in normal and neoplastic human tissues, *Int J Cancer 51*, 522-529.

47. Byfield, J. E. (1989) 5-Fluorouracil radiation sensitization--a brief review, *Invest New Drugs 7*, 111-116.

48. Lawrence, T. S., Tepper, J. E., and Blackstock, A. W. (1997) Fluoropyrimidine-Radiation Interactions in Cells and Tumors, *Semin Radiat Oncol 7*, 260-266.

49. Kievit, E., Bershad, E., Ng, E., Sethna, P., Dev, I., Lawrence, T. S., and Rehemtulla, A. (1999) Superiority of yeast over bacterial cytosine deaminase for enzyme/prodrug gene therapy in colon cancer xenografts, *Cancer Res 59*, 1417-1421.

50. Ireton, G. C., Black, M. E., and Stoddard, B. L. (2003) The 1.14 A crystal structure of yeast cytosine deaminase: evolution of nucleotide salvage enzymes and implications for genetic chemotherapy, *Structure 11*, 961-972.

51. Ireton, G. C., McDermott, G., Black, M. E., and Stoddard, B. L. (2002) The structure of Escherichia coli cytosine deaminase, *J Mol Biol 315*, 687-697.

52. Kievit, E., Nyati, M. K., Ng, E., Stegman, L. D., Parsels, J., Ross, B. D., Rehemtulla, A., and Lawrence, T. S. (2000) Yeast cytosine deaminase improves radiosensitization and bystander effect by 5-fluorocytosine of human colorectal cancer xenografts, *Cancer Res 60*, 6649-6655.

53. Korkegian, A., Black, M. E., Baker, D., and Stoddard, B. L. (2005) Computational thermostabilization of an enzyme, *Science 308*, 857-860.

54. Rogulski, K. R., Kim, J. H., Kim, S. H., and Freytag, S. O. (1997) Glioma cells transduced with an Escherichia coli CD/HSV-1 TK fusion gene exhibit enhanced metabolic suicide and radiosensitivity, *Hum Gene Ther 8*, 73-85.

55. Aghi, M., Kramm, C. M., Chou, T. C., Breakefield, X. O., and Chiocca, E. A. (1998) Synergistic anticancer effects of ganciclovir/thymidine kinase and 5-

fluorocytosine/cytosine deaminase gene therapies, *J Natl Cancer Inst 90*, 370-380.

56. Uckert, W., Kammertons, T., Haack, K., Qin, Z., Gebert, J., Schendel, D. J., and Blankenstein, T. (1998) Double suicide gene (cytosine deaminase and herpes simplex virus thymidine kinase) but not single gene transfer allows reliable elimination of tumor cells in vivo, *Hum Gene Ther 9*, 855-865.

57. Boucher, P. D., Im, M. M., Freytag, S. O., and Shewach, D. S. (2006) A novel mechanism of synergistic cytotoxicity with 5-fluorocytosine and ganciclovir in double suicide gene therapy, *Cancer Res 66*, 3230-3237.

58. Moore, E. C., and Hurlbert, R. B. (1966) Regulation of mammalian deoxyribonucleotide biosynthesis by nucleotides as activators and inhibitors, *J Biol Chem 241*, 4802-4809.

59. Barton, K. N., Paielli, D., Zhang, Y., Koul, S., Brown, S. L., Lu, M., Seely, J., Kim, J. H., and Freytag, S. O. (2006) Second-generation replication-competent oncolytic adenovirus armed with improved suicide genes and ADP gene demonstrates greater efficacy without increased toxicity, *Mol Ther 13*, 347-356.

60. Freytag, S. O., Barton, K. N., Brown, S. L., Narra, V., Zhang, Y., Tyson, D., Nall, C., Lu, M., Ajlouni, M., Movsas, B., and Kim, J. H. (2007) Replication-competent adenovirus-mediated suicide gene therapy with radiation in a preclinical model of pancreatic cancer, *Mol Ther 15*, 1600-1606.

61. Barton, K. N., Tyson, D., Stricker, H., Lew, Y. S., Heisey, G., Koul, S., de la Zerda, A., Yin, F. F., Yan, H., Nagaraja, T. N., Randall, K. A., Jin, G. K., Fenstermacher, J. D., Jhiang, S., Ho Kim, J., Freytag, S. O., and Brown, S. L. (2003) GENIS: gene expression of sodium iodide symporter for noninvasive imaging of gene therapy vectors and quantification of gene expression in vivo, *Mol Ther 8*, 508-518.

62. Kokoris, M. S., and Black, M. E. (2002) Characterization of herpes simplex virus type 1 thymidine kinase mutants engineered for improved ganciclovir or acyclovir activity, *Protein Sci 11*, 2267-2272.

63. Berenbaum, M. C. (1989) What is synergy?, *Pharmacol Rev 41*, 93-141.

64. Chou, T. C., and Talalay, P. (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors, *Adv Enzyme Regul 22*, 27-55.

65. Tallarida, R. J. (2001) Drug synergism: its detection and applications, *J Pharmacol Exp Ther 298*, 865-872.

66. Vermes, A., Guchelaar, H. J., and Dankert, J. (2000) Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions, *J Antimicrob Chemother 46*, 171-179.

67. Bean, B. (1992) Antiviral therapy: current concepts and practices, *Clin Microbiol Rev 5*, 146-182.

68. Park, J. I., Cao, L., Platt, V. M., Huang, Z., Stull, R. A., Dy, E. E., Sperinde, J. J., Yokoyama, J. S., and Szoka, F. C. (2009) Antitumor therapy mediated by 5-fluorocytosine and a recombinant fusion protein containing TSG-6 hyaluronan binding domain and yeast cytosine deaminase, *Mol Pharm 6*, 801-812.