EVALUATING THE EFFECT OF PRODRUG METABOLISM ON THE BYSTANDER EFFECT IN CANCER GENE THERAPY

By

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EVALUATING THE EFFECT OF PRODRUG METABOLISM ON THE BYSTANDER EFFECT IN CANCER GENE THERAPY

Abstract

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Suicide gene therapy for cancer offers a selective approach to eradicating tumor cells by using non-toxic prodrugs in conjunction with prodrug-activating enzymes expressed at tumor sites. The efficacy of suicide gene therapy is often limited by the lack of a suitable gene delivery system and by the low activity of suicide enzymes towards the prodrug. These limitations may be overcome by using mutant and/or fusion enzymes that display increased activity towards a prodrug or by using enzymes that elicit a strong bystander effect (BE). The BE refers to the capability of transfected cells to induce death in neighboring untransfected cells, typically through intercellular transfer of cytotoxic prodrug metabolites.

The *Escherichia coli* or bacterial cytosine deaminase (bCD) converts the prodrug 5-fluorocytosine (5FC) to the chemotherapeutic agent 5-fluorouracil (5FU). When coupled with *E. coli* uracil phosphoribosyltransferase (bUPRT), the resultant fusion enzyme, bCD/UPRT, induces greater cytotoxicity compared to bCD alone. Several bCD mutants generated in our laboratory also increase cytotoxicity relative to bCD. One mutant, bCD_{1525}, was coupled with bUPRT in an attempt to augment cell death.

It was hypothesized that expression of this fusion enzyme, bCD_{1525}/UPRT, would enhance 5FC metabolism, thus generating higher concentrations of cytotoxic metabolites. We predicted that altered metabolite concentrations would shift the BE mechanisms away from 5FU diffusion (the mechanism typically reported for the 5FC/bCD system). We anticipated different mechanisms, namely transfer of apoptotic vesicles and metabolite movement through gap junctions, to play a more central role in the BE of cells expressing fusion enzymes.

To examine 5FC metabolism, we developed two novel high-performance liquid chromatography (HPLC) methods, one to quantify 5FC anabolic products produced by suicide enzymes, the other to quantify a 5FC catabolic product generated by endogenous mammalian enzymes. Additionally, we examined the contribution of apoptotic vesicles to the BE and assessed the ability of our cancer cell lines to transfer compounds through gap junctions. Taken together, these data provide a more complete understanding of the relationship between prodrug metabolism, cytotoxicity, and the BE. This knowledge will aid in future endeavors to improve enzymes for more effective suicide gene therapy.

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CHAPTER ONE

Introduction

As researchers learn more about the genetic defects responsible for the onset of deadly or debilitating diseases, scientific questions arise as to whether correction of the culprit genes would result in treatment, or even reversal, of disease states. This treatment strategy, known as gene therapy, seeks to correct genetic diseases by introducing into cells or tissues functional gene copies to replace or supplement defective or missing genes[1].

For gene therapy to be practical, it must provide a one-time treatment strategy able to produce a therapeutic effect over the entire life of the patient. Introduction and maintenance of genetic material within patients remains the major limitation to effective gene therapy. Delivery of therapeutic DNA poses several obstacles including: 1) delivery of the genetic material to the correct tissues/cells; 2) transportation of the genetic material to the nucleus; 3) expression of functional protein following gene transcription; and 4) adequate duration of gene and/or protein expression to result in correction of the patient's disease[1].

Delivery Systems for Gene Therapy

Currently studied delivery systems for gene therapy can be divided into two categories, viral and non-viral, but problems associated with each limit their ability to provide safe and efficient delivery of therapeutic genes[2]. Effective delivery systems

must be able to overcome a variety of extracellular and intracellular barriers including: 1) entering the host cells, 2) releasing genetic material into the cytosol, and 3) for transcription, promoting transport of the genetic material from the cytosol to the nucleus[3].

Viral vectors are the most commonly studied gene delivery vehicles and are considered superior to non-viral vectors due to their natural ability to introduce their genetic material into target cells. Viral vectors can be further divided into two classes: those that integrate their genome into the host genetic material and those that remain as extrachromosomal episomes. Integrating and non-integrating viral vectors have been used in clinical gene therapy trials, though with limited therapeutic success and sometimes fatal side-effects. Commonly used viral vectors include non-integrating adenovirus, adeno-associated virus, herpes simplex virus, and integrating retrovirus and lentivirus. The use of different viral vectors provides diversity in carrying capacity, tropism, and potential immune responses of the patient (Table 1.1). Carrying capacity refers to the amount of non-viral genetic material the vector can carry. Tropism refers to the natural preference of a virus to infect certain tissue or cell types; this preference typically results from the ability of the cell or tissue to support viral entry or growth[4]. While some viruses naturally infect many cell or tissue types (the adenovirus enters any cell that express the Coxsachievirus-Adenovirus Receptor (CAR)), others are more specific (herpes viruses target neurons while retroviruses require actively dividing cells for replication)[5, 6]. Adding targeting ligands to vectors may change their tropism; it was demonstrated that attaching the ligand fibroblast growth factor 2 to adenovirus vectors increased their specificity for cancers that over-express the fibroblast growth factor

receptor[7, 8]. Additionally, adding a targeting motif may reduce toxicity associated with non-specific vector binding[9]. Toxicity can also be reduced by removing viral genes responsible for virus replication, which also increases vector carrying capacity[10, 11].

Vector	Duration	Tropism	Immunogenic Potential	Carrying Capacity
Adenovirus (Ad)	Transient	Broad; Cells expressing CAR receptor	High	7 kb
Adeno-associated virus (AAV)	Mostly transient; integration <10%	Broad	Low	5 kb
Herpes Simplex Virus (HSV)	Transient	Neurons	High	20 kb
Retrovirus	Stable via integration	Dividing cells	Low	9 kb
Lentivirus	Stable via integration	Dividing and non-dividing cells	Low	10 kb

Table 1.1. Summary of commonly used viral vectors for gene therapy. Adapted from Hatefi *et al*.[9]

Limitations associated with viral vectors have lead to a surge in efforts to develop non-toxic, efficient, and cost-effective non-viral vectors. Current non-viral delivery strategies include lipid-based vectors (liposomes), protein polymers, episomal plasmids, and injection of naked DNA into affected tissues[9]. However, these vectors are associated with their own limitations. Plasmids and naked DNA work best in dividing cells but do not offer long-term gene expression. Liposomes provide high transfection efficiency but can be cytotoxic[12] while polymers, though biocompatible, exhibit poor gene-transfer efficiencies[3]. Research is currently being conducted to improve both viral and non-viral vectors in terms of homing to specific cells, improving gene transfection efficiency, reducing systemic toxicity, and increasing duration of gene expression.

ADDITIVE/REPLACEMENT GENE THERAPY

Gene therapy strategies that introduce functional genes meant to replace or supplement the function of defective genes are known as *additive/replacement therapy*. This treatment offers the promise of curing monogenic, inherited diseases such as hemophilia, sickle cell anemia, cystic fibrosis, and primary immunodeficiency diseases (PIDs)[13]. PIDs are genetic conditions that affect the function of specific immune cells and are traditionally treated by hematopoietic stem cell transfer; however, this treatment is less than ideal since success relies on identifying an HLA-identical match and even then carries the risk of graft-versus-host disease[14]. Therefore, researchers are considering gene therapy as a suitable alternative treatment. PIDs that are attractive candidates for gene therapy include the severe combined immunodeficiency disorders (SCIDs), rare disorders resulting in severe defects in the T- and B-lymphocyte maturation. Without treatment, SCID patients typically die within the first year of life due to severe recurrent infections[14].

Use of gene therapy to treat SCIDs has been evaluated in numerous clinical trials. In 1990, an autosomal-recessive form of SCID resulting from the deficiency of adenosine deaminase (ADA), an enzyme of the purine salvage pathway, was the focus of the first ever gene therapy trial. A normal ADA gene was introduced *ex vivo* in the patients' T-lymphocytes and the altered cells were re-introduced. The relevant success of this clinical trial remains controversial since only one of the two patients experienced any therapeutic benefit. Although transduced T cells have persisted for over a decade in this patient, bovine adenosine deaminase conjugated to polyethylene-glycol (PEG-ADA) continue to be administered to this patient, raising the question if this is 'true' gene

therapy[15]. In 1999, doctors used gene therapy to treat a patient suffering from ornithine transcarbamylase deficiency (OTCD). This disorder manifests when OTC, a liver enzyme that removes toxic ammonia from the body, is non-functional. During the trial, adenovirus vectors carrying genes encoding functional OTC were introduced to patients via hepatic vein injection. Contrary to researchers' predictions, the adenovirus entered the general circulation, triggering a severe immune response against one patient's organs, ultimately resulting in fatal multi-organ system failure[13].

In April 2000, a gene therapy trial was performed to treat children suffering from X-linked severe combined immunodeficiency disorder (X-SCID), the most common form of SCID. X-SCID patients lack the gamma receptor subunit for cytokine IL-2, necessary for thymic development, B cell class-switching, and maturation of natural killer cells[16]. While 9 of the 10 patients showed evidence of a reconstituted immune system, 2 of the children were later diagnosed with T-cell leukemia. Findings from a biomedical review panel indicated that while insertion of the *IL2Rγc* gene into patient genomes via the retroviral vector resulted in a therapeutic effect, it also caused insertional mutagenesis in the children diagnosed with leukemia due to integration near the T-cell oncogene *LMO2*[17].

These clinical trials failed largely due to the lack of a suitable gene delivery system. As mentioned, a great deal current research focuses on improving delivery systems to better target cells/tissues of interest, to increase the duration of gene expression, and to bolster safety. However, until researchers adequately address these issues, hurdles to providing effective additive/replacement gene therapy will remain.

ABLATION GENE THERAPY

Gene therapy is not limited to inherited diseases but may also be used to treat acquired diseases such as cancer. Cancer is a disease characterized by the presence of abnormal cells and uncontrolled cell division[4]. It is second only to heart disease as a leading cause of death in the United States and in 2008 was responsible for approximately 566,000 deaths[18]. Two basic goals of cancer treatment include: 1) controlling local tumor growth by disrupting neoplastic cell growth, or 2) removing neoplastic tissues from the body. This second goal may be realized through a different type of gene therapy termed *ablation therapy*. In ablation therapy, unwanted cells are targeted for destruction. Unlike addition/replacement therapy, disease targets of ablation therapy are not necessarily monogenic. Additionally, ablation therapy is meant to provide a transient therapeutic effect, making it a more realistic treatment method than long-term addition/replacement gene therapy. Traditional therapies targeting the destruction or removal of cancerous cells include surgery, irradiation, and chemotherapy. No single treatment strategy is 100% effective, and each method poses specific challenges that inhibit its success; surgery is limited by tumor size, location, and morphology, while both irradiation and chemotherapy result in the disruption of healthy tissues along with cancerous ones. Furthermore, chemotherapy is often associated with many unpleasant and dose-limiting side effects for patients, including nausea, lethargy, anemia, alopecia, and pain. For these reasons, researchers have begun to focus their attention on developing new, less invasive cancer treatment alternatives, such as ablation gene therapy, also known as gene-directed enzyme prodrug gene therapy (GDEPGT) or suicide gene therapy. Suicide gene therapy was first described

by F.L. Moolten in 1986[19]. He proposed that cancerous cells could be genetically modified to render them sensitive to a prodrug by introducing into cells a gene encoding an enzyme with the ability to convert an inactive prodrug to a cytotoxic product (Figure 1.1). In most cases, this product interferes with DNA and/or RNA synthesis to bring about cell death[20].

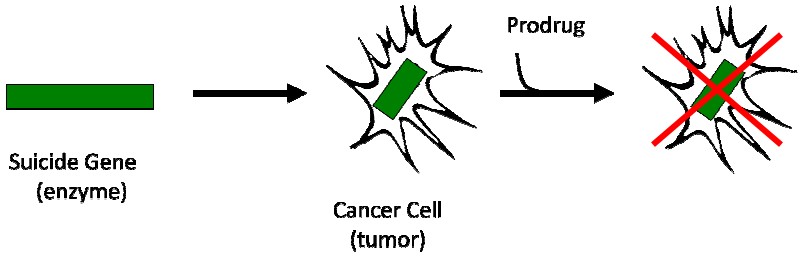

Figure 1.1. Overview of suicide gene therapy.

THE BYSTANDER EFFECT

The bystander effect (BE), also originally described by F.L. Moolten, refers to the transfer of toxic metabolites from the transfected cells into non-transfected neighbor cells. This metabolite transfer results in high levels of tumor cell killing even when the suicide gene is introduced into as few as 10% of tumor cells[19] (Figure 1.2). The BE, thereby, compensates for commonly low transfection efficiencies, which significantly hinder gene therapy effectiveness. The BE contributes to complete tumor eradication by increasing the percentage of tumor cells killed in a single treatment. This will theoretically decrease the need for multiple rounds of therapy, thus lowering the potential of host side effects associated with repeated prodrug administration.

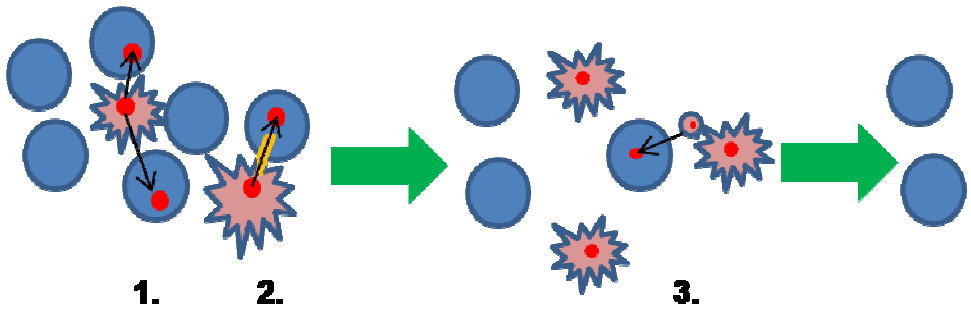

Figure 1.2. Overview of the bystander effect.
The mechanisms of the BE include intercellular diffusion of cytotoxic metabolites (1), metabolite movement through gap junctions (2) or transfer of apoptotic vesicles (3).

The BE manifests via different mechanisms. Small, uncharged metabolites move between cells via diffusion through the cellular membrane. In contrast, phosphorylated metabolites contain bulky, partially ionized groups, making them unable to diffuse to neighboring cells[21]. Transfer of these metabolites occurs through gap junctions created between contacting cells[22]. Gap junctions are channels between communicating cells that allow passage of ions and small molecules between the cells' cytoplasmic regions. These structures are composed of six four-pass transmembrane proteins (connexins) and are 2-4 nm in length and a maximum of 1.5 nm in diameter[23].

For a long time, gap junctions were considered the sole means by which phosphorylated metabolites moved between cells[21]. However, certain cancers such as breast cancer down-regulate the formation of gap-forming cells, resulting in disordered or low numbers of gap junctions[24]. Other mechanisms, therefore, may contribute to bystander cell killing in these cells[25]. In 1993, Freeman *et al.* demonstrated that suicide enzyme-expressing cells confer their toxicity to non-suicide enzyme-expressing cells following their death. This led to the hypothesis that when suicide enzyme-expressing cells undergo apoptosis, they release vesicles that are phagocytized by

neighboring cells, resulting in the transfer of cytotoxic metabolites or apoptotic signals to and subsequent death of non-suicide enzyme-expressing cells[26]. However, the extent to which transfer of apoptotic vesicles contributes to the BE remains unclear.

In addition to this so-called "local" BE, a distant BE has been reported in *in vivo* mouse models. For example, following prodrug treatment of RM1 murine prostate cancer cells expressing suicide enzymes, Khatri *et al*. observed increased tumor cell necrosis coupled with inhibited growth of lung metastases, suggesting that the immune system may hinder growth of tumor cells at sites distant from the primary tumor[27]. Additionally, the presence of inflammatory infiltrates, chemokines, and cytokines has been reported in regressing tumors of immunocompetent mice undergoing suicide gene therapy treatment, suggesting a role for the immune system in eliciting a BE[28-34].

SUICIDE ENZYME/PRODRUG SYSTEMS

Many genes are being considered for use in suicide gene therapy, the most widely studied of which are Herpes Simplex Virus thymidine kinase (HSVTK), bacterial cytosine deaminase (bCD), and yeast cytosine deaminase (yCD). Using non-mammalian enzymes limits activity to transfected cells, therefore allowing selective eradication of cancerous cells while leaving healthy cells relatively unharmed, an outcome not achievable with irradiation or chemotherapy. However, by introducing non-human enzymes into the body, there is a risk of eliciting an immune response[35]. Currently, a few human-derived enzymes are also being investigated for potential use as suicide enzymes, including human thymidine kinase-2 and deoxycytidine kinase (Table 1.2)[21].

Enzyme	Source	Natural Substrate	Prodrug	Drug Mechanism of Action
Herpes Simplex Virus Thymidine Kinase (HSVtk)	*Herpes Simplex Virus 1*	Thymidine	GCV, ACV	DNA synthesis inhibition
Cytosine Deaminase (CD)	*Escherichia coli* *Saccharomyces cerevisiae*	Cytosine	5-FC	DNA synthesis inhibition, RNA damage, DNA damage
Drosophila melanogaster Deoxyribonucleoside Kinase (*Dm*-dNK)	*Drosophila melanogaster*	Deoxynucleosides	AZT, ddC, CdA, GCV, AraG, ddT F-AraA	DNA synthesis inhibition DNA synthesis inhibition, Ribonucleotide reductase inhibition
Deoxycytidine Kinase (dCK)	*Homo sapiens*	Deoxycytidine	dFdC, AraC, AZT	DNA synthesis inhibition, Ribonucleotide reductase inhibition
Purine Nucleoside Phosphorylase (PNP)	*Escherichia coli*	Purine ribonucleosides	Me(talo)MeP-R	RNA/protein synthesis inhibition

Table 1.2. Commonly studied suicide enzyme/prodrug systems and their mechanisms of action.

Cytosine Deaminase

Cytosine deaminase (EC 3.5.4.1) derived from *E. coli* (bCD) or *S. cerevisiae* (yCD) is a commonly used suicide enzyme. It catalyzes the hydrolytic deamination of the prodrug 5′-fluorocytosine (5FC) to produce the chemotherapeutic agent 5′-fluorouracil (5FU). 5FU is converted by endogenous mammalian enzymes to 5′-fluorouridine (5FURD), then subsequently to the cytotoxic metabolites 5′-fluorouridine monophosphate (5FUMP) and 5′-fluoro-2′deoxyuridine monophosphate (5FdUMP) (Figure 1.3). 5FdUMP competes with 2′deoxyuridine monophosphate (dUMP) for the binding site in thymidylate synthase (TS), an enzyme that catalyzes the transfer of a methyl group from folate 5,10-methylenetetrahydrofolate (CH_2THF) to the 5′ carbon of dUMP. When 5FdUMP is substituted for dUMP, the presence of a fluorine atom at the 5′ site results in tighter substrate binding, prohibiting methyl transfer and inhibiting TS activity[36]. By preventing TS-mediated formation of thymidine monophosphate from dUMP, subsequent generation of thymidine triphosphate (dTTP) is inhibited (Figure

1.3). Disruption of this reaction impairs DNA synthesis and repair by reducing dTTP availability and upsetting the balance of deoxynucleotide (dNTP) pools[37-39].

Inhibition of TS by 5FdUMP binding is considered the primary mechanism of 5FC cytotoxicity, although other 5FC metabolites may contribute to cellular damage following misincorporation into DNA or RNA (Figure 1.3). 5FdUTP can be a substrate for DNA polymerase, resulting in its incorporation into growing DNA strands. Removal of this base results in a single-strand breaks that may be difficult to repair due to dTTP depletion[36]. 5FUTP is incorporated into RNA via RNA polymerase, thus impairing normal processing of pre-rRNA and splicing of pre-mRNA. Additionally, 5FU may replace uracil residues in RNA, resulting in decreased mRNA polyadenylation and formation of a covalent complex of tRNA and enzymes involved in uracil post-translational modification[36].

Though bCD and yCD both catalyze the hydrolytic deamination of 5FC, these two enzymes appear to be evolutionarily distinct. Both rely on a catalytic ion for maximum activity, but bCD is comprised of a hexamer of 48-kDa subunits and uses a coordinated iron (II) ion whereas yCD assembles into a homodimer from 17.5-kDa subunits and relies on a catalytic zinc ion[41-43]. bCD belongs to the α/β TIM barrel fold family and is most structurally similar to human adenosine deaminase while yCD belongs to the amidohydrolase protein fold family and is more similar to bacterial and eukaryotic cytidine deaminases than to bacterial cytosine deaminase [35, 39].

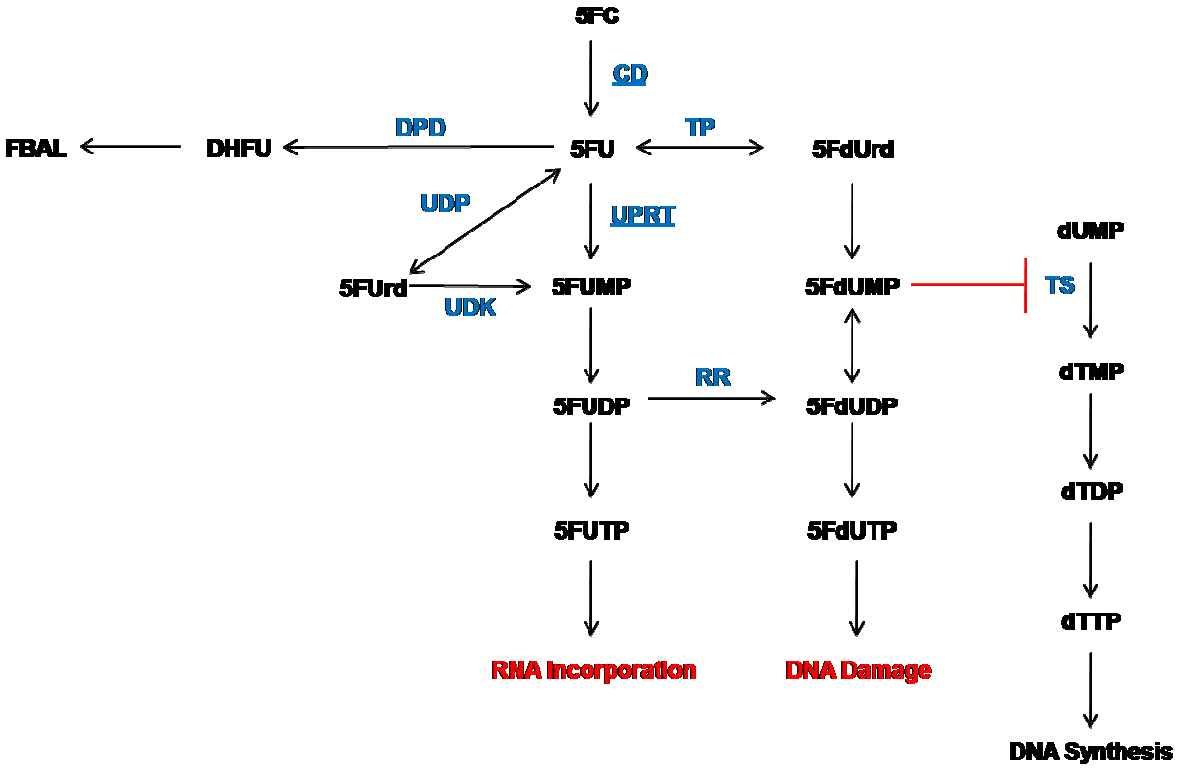

Figure 1.3. Activation pathway of 5-fluorocytosine in mammalian cells transfected with bacteria-derived suicide enzymes, adapted from Longley, *et al*.[40]

Non-mammalian enzymes are underlined. CD: cytosine deaminase; UPRT: uracil phosphoribosyltransferase; DPD, dihydropyrimidine dehydrogenase; TP: thymidine phosphorylase; TS: thymidylate synthase; UDP: uridine phosphorylase; UDK: uridine kinase; RR: ribonucleotide reductase; 5FC: 5-fluorocytosine; 5FU: 5-fluorouracil; DHFU: dihydrofluorouracil; FBAL: 2-fluoro-β-alanine; 5FURD: 5-fluorouridine; 5FUMP: 5-fluorouridine monophosphate; 5FUDP: 5-fluorouridine diphosphate; 5FUTP: 5-fluorouridine triphosphate; 5FdURD: 5-fluorouridine; 5FdUMP: 5-fluorodeoxyuridine monophosphate; 5FdUDP: 5-fluorodeoxyuridine diphosphate; 5FdUTP: 5-fluorodeoxyuridine triphosphate; dUMP: deoxyuridine monophosphate; dTMP: deoxythymidine monophosphate; dTDP: deoxythymidine diphosphate; dTTP: deoxythymidine triphosphate.

As CD displays relatively low activity toward 5FC compared to cytosine, its natural substrate, high levels of prodrug must be administered for tumor eradication[39, 44]. However, because bacteria comprising the normal flora of the gastrointestinal tract convert 5FC to 5FU, patients may experience hematologic and gastrointestinal toxicity[35, 45]. In order to address this issue, current research in our laboratory focuses on using mutagenesis to shift the substrate preference of these enzymes.

ENZYME ENGINEERING

We use enzyme engineering strategies to improve enzyme activity towards the prodrug with the goal of reducing the prodrug dose needed to achieve complete tumor ablation. Enzyme engineering entails performing mutagenesis on enzymes then screening the mutants via genetic complementation and plate assays in order to select for improved variants. This strategy has produced mutants of HSVTK, bCD, and yCD, among others, that display improved cell killing ability both *in vitro* and *in vivo*[46-51]. We have described a number of mechanisms of enzyme improvement including: 1) directly increasing the enzyme's ability to bind prodrug, 2) increasing catalytic activity, 3) increasing enzyme stability, and 4) decreasing the enzyme's ability to bind native substrate resulting in lowering prodrug and native substrate competition in the enzyme's active site.

Mutagenesis/Selection Strategies

We use several mutagenesis strategies to generate enzyme variants, including site-directed mutagenesis, computational design, and random mutagenesis. As the name suggests, site-directed mutagenesis introduces substitutions at specific amino acid residues within an enzyme. Regions chosen for site-directed mutagenesis are often based on the enzyme crystal structures coupled with molecular modeling studies or DNA sequence alignments with homologous enzymes. Unfortunately, these data are not always available, limiting the application of this strategy. Computational design uses computer modeling coupled with X-ray structural data to determine amino acids

critical for enzyme function, folding, or stability. These amino acids are then selectively changed and activity assessed.

Random mutagenesis entails introducing mutations throughout a selected gene region (regio-specific mutagenesis) or over the entire open reading frame (error-prone PCR). This allows for greater flexibility in amino acid substitutions than does targeted mutagenesis. Additionally, this technique can be applied in the absence of enzyme structural data and has the potential to introduce combinations of amino acid substitutions that are optimal for enhanced enzyme activity but are not obvious from molecular modeling studies. Regio-specific random mutagenesis utilizes over-lapping, complimentary oligonucleotides containing random nucleotides at specific sites or having a limited percent randomness[52-54].

Following mutagenesis, enzymes are screened for activity toward the natural substrate (positive selection) and the prodrug (negative selection) using genetic complementation and plate assays. Genetic complementation employs bacterial or yeast strains lacking the enzyme activity of interest. This gene (wild-type or variant) is then introduced and activity is assessed under selection conditions that enable growth (positive selection) or restrict growth (negative selection) of cells expressing functional enzymes. For example, to screen mutant bCD enzymes, the lysogenized *E. coli* strain GIA39(DE3), deficient in CD and orotidine 5′-phosphate decarboxylase activities, was transformed with the expression vector pETHT carrying wild-type or mutant bCD. These cells were then grown on positive or negative selection plates. Growth was compared to growth of cells expressing wild-type enzymes or empty plasmid. Positive selection plates contained cytosine as the sole pyrimidine source, and so growth on

these plates indicated expression of a functional bCD enzyme. Negative selection plates were supplemented with 5FC, and so expression of a functional bCD enzyme resulted in conversion of 5FC to 5FU, resulting in cell death.

When screening mutants, different prodrug concentrations are used to compare activity of mutants. For example, plates containing high prodrug concentrations are used in initial screening assays. Mutants that show increased activity at high prodrug concentrations relative to wild-type (i.e., induce cell death) are then streaked onto plates containing progressively lower prodrug concentrations. Enzymes that restrict growth at the lowest concentrations are designated as being “super sensitive” to the prodrug.

Improving yCD

Compared to bCD, wild-type yCD displays relatively good activity toward 5FC, with a K_m value 22-fold lower than bCD[55]. However, side effects resulting from therapeutic 5FC doses continue to be an obstacle for treatment of patients. In an attempt to further shift yCD substrate preference toward 5FC, our laboratory introduced mutations into the enzyme active site using regio-specific random mutagenesis. Through these experiments, three yCD mutants able to confer sensitivity to 5FC in *E. coli* were isolated. These enzymes were then ligated into the mammalian expression vector pcDNA and were introduced into C6 rat glioma cells via transfection. The IC_{50} value, or the amount of drug needed to kill half of the cells, was then determined (Table 1.3). The yCD mutant containing the amino acid substitution D92E displayed a 30% reduction in IC_{50} value compared to wild-type yCD[56].

Although yCD shows more activity toward 5FC than does bCD, it is thermolabile. At 50 °C, the half-life of yCD is ~4 hours, a property which may limit yCD efficacy for therapeutic use compared with bCD, which displays a half-life of >100 hours at 50 °C[55, 57]. To address this issue, our laboratory created thermostabilized yCD mutants through computational design. The variants were named based on the number of amino acid substitutions found within the hydrophobic core; the enzyme with two mutations (A23L/I140L) was designated yCD_{double} and that with three (A23L/I120L/V108I) named yCD_{triple}. Kinetic studies demonstrated that the variants displayed slightly reduced V_{max} and K_m values but no appreciable change in catalytic efficiency (k_{cat}/K_m) relative to wild-type yCD. However, the thermostability of the variants increased compared to wild-type, with yCD_{double} displaying a half-life of ~21 hours and yCD_{triple} ~117 hours at 50 °C[51]. Additionally, the IC_{50} values decreased approximately 30% and 48% for yCD_{double} and yCD_{triple}, relatively, compared to wild-type yCD[56] (Table 1.3).

	5FC IC_{50} Values (mM)
pcDNA	N/A
yCD	11.4
D92E	8.0
yCD_{Double}	8.0
yCD_{Triple}	6.0

Table 1.3. 5FC IC_{50} values (mM) of C6 cells expressing yCD enzymes as determined by *in vitro* cytotoxicity assays.

Improving bCD

With the aim of generating bCD variants with increased catalytic activity toward 5FC, alanine-scanning and regio-specific random mutagenesis studies of bCD were

conducted. The alanine-scanning study used site-directed mutagenesis to change residues 310-320 lining the cytosine-binding pocket of the bCD active site to alanine. Seven active mutants were identified, though only D314A conferred increased 5FC sensitivity (17-fold) and decreased cytosine activity (2-fold) in genetic complementation studies[58]. Results from another mutagenesis study revealed that substitution of a small, uncharged amino acid (glycine or serine) at residue 314 produced functional enzymes, whereas substitution of residues with basic side-chains (lysine, arginine, or histidine) yielded inactive enzymes, as assessed by genetic complementation[59]. The data from kinetic studies revealed that while D314A displayed a 19-fold increase in relative activity for 5FC over cytosine as compared with wild-type bCD, D314G and D314S only displayed a 2.5 and 4.2 increase in relative activity, respectively[59].

The regio-specific random mutagenesis study targeted a second group of residues lining the bCD active site, 149-159, in addition to the 310-320 residues[50]. Mutant enzymes were introduced into *E. coli* and genetic complementation assays performed as previously published[58]. Twelve variants displayed increased sensitivity to 5FC and were isolated and sequenced. Enzyme assays demonstrated that three of these mutants, bCD_{1246}, bCD_{1525}, and bCD_{1779}, converted high levels of 5FC substrate so these enzymes were purified for kinetic analysis. These three mutants displayed reduced activity towards cytosine and increased k_{cat} values for 5FC (Table 1.4), resulting in an overall shift in substrate preference for 5FC over cytosine by 18- to 19-fold compared to bCD [50].

	Wild-type bCD		bCD1246		bCD1525		bCD1779	
	Cytosine	5FC	Cytosine	5FC	Cytosine	5FC	Cytosine	5FC
K_m(mM)	0.46	3.76	1.65	6.73	4.9	12.69	3.16	7.55
k_{cat}(sec^{-1})	49.68	19.71	2.13	83.68	1.69	101.74	3.98	115.24
k_{cat}/ K_m(mM^{-1} sec^{-1})	106.85	5.14	1.29	12.44	0.35	8.02	0.94	15.27
Relative efficiency to the wild-type	1	1	0.01	2.37	0.003	1.53	0.0009	2.91
Relative substrate specificity	0.95	0.005	0.09	0.91	0.04	0.96	0.06	0.94
Relative substrate specificity to the wild-type	1	1	0.09	18.2	0.04	19.2	0.05	18.8

Table 1.4. Catalytic activities of the wild-type and mutant bCD with cytosine and 5FC[50]

Sequence data revealed that, unexpectedly, only one of the three "best" mutants contained a substitution at D314, the position previously identified as a key mutation to incur 5FC sensitivity. However, all three of these mutants contained the amino acid substitution D317G and an additional substitution at residue F316. To elucidate which of these substitutions were critical in conferring 5FC sensitivity, site-directed mutagenesis was used to generate the following bCD mutants: F316L, F316C, F316V, D317G, F316L/D317G, F316C/D317G and F316V/D317G. The activity of these mutants was compared with the activity of parent mutant enzymes (bCD_{1246}=D314E/F316L/D317G, bCD_{1525}=V152A/F316C/D317G and bCD_{1779}=V315L/F316V/D317G), using *E. coli* complementation assays, as described above. Mutants containing the substitution D317G displayed increased sensitivity at 20 µg/ml 5FC relative to wild-type bCD. However, introduction of this substitution alone did not confer sensitivity at 0.5 µg/ml 5FC relative to parent mutant enzymes. A minimum of two substitutions (F316 and D317G) were needed to sensitize cells to low levels of 5FC (Table 1.5). The reason for this may be elucidated by examining the structure of

bCD. Structural studies of wild-type bCD and variants illustrated that substituting amino acids with smaller side chains at positions D314 or D317 opened a pocket in the region where the fluorine atom of 5FC binds[50, 59].

Further evaluation of *in vitro* prodrug sensitivity demonstrated that one mutant, bCD_{1525}, was more sensitive to 5FC than the other mutants. HCT116 (human colorectal cancer) cells expressing bCD_{1525} displayed an IC_{50} value of approximately 1.5 mM 5FC, a 20-fold reduction relative to wild-type bCD[50]. Additionally, *in vivo* analysis using a xenograft tumor mouse model of transfected HCT116 cells demonstrated significant suppression of tumor growth when cells expressed bCD_{1525} enzyme compared to wild-type bCD and the mice treated with 5FC intraperitoneally[50] (Figure 1.4).

Mutation	Cytosine	5FC (0.5 µg/ml)	5FC (20 µg/ml)
pETHT	-	-	-
bCD	++	++	++
D317G	++	+	_
bCD_{1246}	++	+	_
F316L	++	++	+
F316L/D317G	++	++	+
bCD_{1525}	++	+	+
F316C	++	++	+
F316C/D317G	++	+	_
bCD_{1779}	++	+	_
F316V	++	++	++
F316V/D317G	++	+	_

Table 1.5. Summary of bCD mutant growth on selection plates.
++, growth; +, slightly inhibited growth; -, no growth.

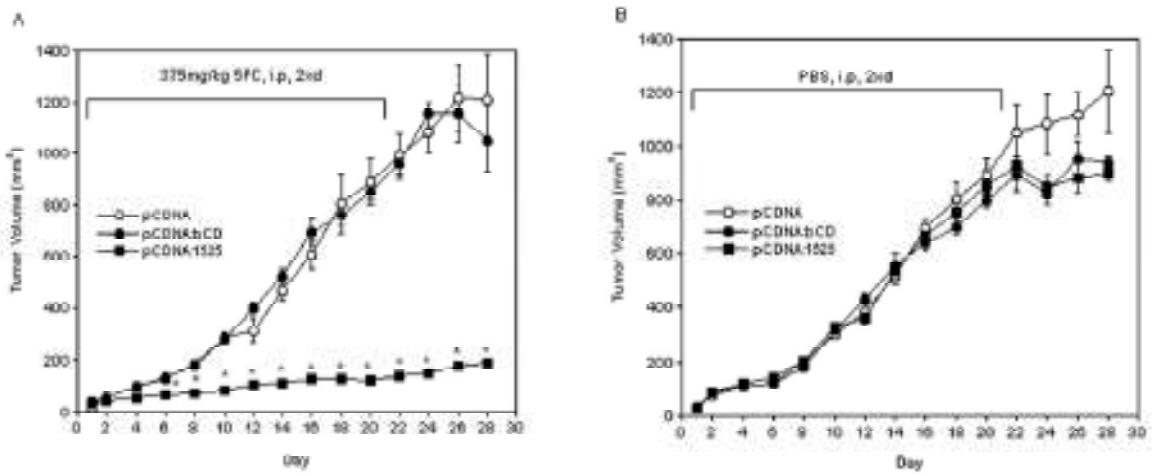

Figure 1.4. Tumor growth during and after 5FC treatment in a xenograft model[50].

By using enzyme engineering strategies, we have generated bCD and yCD variants that display improved tumor cell killing ability at prodrug doses lower than needed to achieve similar cell killing using wild-type enzymes. Additionally, the yCD variants demonstrate improved thermostability. These improvements are necessary to provide a clinically applicable therapy since they will presumably allow for lower prodrug dosages (potentially decreasing negative patient side effects) and a more stable suicide enzyme at physiological temperatures.

To further characterize these mutants in terms of prodrug metabolism, we used reverse-phase high-performance liquid chromatography (HPLC). Chapter 2 describes the validation of an isocratic HPLC method we developed to detect and quantify 5FC and its four major anabolites (5FU, 5FURD, 5FUMP, and 5FdUMP). This method was used to evaluate 5FC metabolism in cells expressing wild-type and mutant bCD enzymes. We also used this method to assess the differences in cell-line sensitivity toward 5FC. Additionally, this method will be used as part of a comprehensive

evaluation of bCD and yCD activity aimed at determining a “superior mutant enzyme” for use in suicide gene therapy.

PATHWAY ENGINEERING

Another approach to improving enzymes for use in suicide gene therapy is “pathway engineering,” or coupling the enzymes responsible for catalyzing consecutive steps in the prodrug activation pathway. In mammalian cells, the first step in prodrug activation, catalyzed by suicide enzymes, is often the rate-limiting step due to low catalytic activity of suicide enzymes. However, it is hypothesized that when enzyme variants with improved activity towards a prodrug are expressed in mammalian cells, these improved enzymes may generate high concentrations of primary metabolites, thus, shifting the rate-limiting step from activation of the prodrug to conversion of the first metabolite to the second metabolite (often catalyzed by endogenous enzymes). This may result in a “metabolic bottleneck”[60]. By using pathway engineering to introduce a second enzyme able to act upon the intermediate metabolites, the overall rate of prodrug conversion to cytotoxic compounds may be expedited.

While expression of suicide fusion enzymes has been shown to increase cell death following prodrug administration, the exact molecular mechanisms of this improvement are unknown. Several possible mechanisms may explain the improved cell killing effects, including proximity of the enzyme active sites, substrate shunting along electrostatic barriers created by charges present on the protein surface, or increased probability of the second enzyme contacting the substrate due to increased local concentration of enzymes. In each of these cases, the amount of intermediate

metabolites able to diffuse into the cellular environment would be limited, allowing the second enzyme of the metabolic pathway to convert the initial intermediate metabolite to the next product.

Pathway engineering may also help eliminate degradation of intermediate metabolites by endogenous enzymes. A prime candidate for this is the CD/5FC system. The endogenous mammalian enzyme dihydropyrimidine dehydrogenase (DPD; EC 1.3.1.2) reversibly reduces the 5, 6 double bond of 5FU, resulting in the formation of dihydrofluorouracil, which is further degraded to 2-fluoro-β-alanine (FBAL). FBAL is non-cytotoxic and readily excreted in patient urine; it is estimated that over 80% of systemically administered 5FU is excreted as FBAL[61, 62] (Figure 1.3). Levels of DPD can vary significantly between patients and can be up- or down-regulated in different tissues and cancer types. Up-regulation of DPD has been observed in colorectal, prostate, and head-and-neck cancers, resulting in increased FBAL production and decreased 5FU elimination half-life and efficacy[61, 62]. Alternatively, for patients harboring inactive variants of DPD, 5FU treatment is extremely toxic and potentially fatal[63].

DPD deficiency is an autosomal recessive metabolic disorder that affects an estimated 3-5% of the general population, though the incidence of deficiency varies widely between ethnic groups[64]. Partial or complete DPD deficiency is responsible for approximately 30-57% of severe 5FU-related toxicities, including cardiac toxicity, mucositis, myelosuppression, diarrhea, and death[61, 65]. Single nucleotide polymorphisms (SNPs) within the *DPYD* gene encoding the DPD enzyme are the most common reason for non-functional DPD and over 30 SNPs have been identified[65].

Additionally, epigenetic factors and aberrant methylation in the *DPYD* promoter region are potential causes of non-functional DPD[66].

One approach for limiting 5FU availability to DPD is to use pathway engineering to improve the 5FC/CD system. The bacterial or yeast enzyme uracil phosphoribosyltransferase (UPRT; EC 2.4.2.9) is part of the prokaryotic pyrimidine salvage pathway and is encoded by the *upp* gene *in E. coli* and the *FUR1* gene in *S. cerevisiae*[67, 68]. Bacterial UPRT (bUPRT), a 78 kDa protein, and yeast UPRT (yUPRT), a 40 kDa protein, catalyze the transfer of a ribosyl-phosphate group to uracil to form uridine monophosphate (UMP)[68-70]. UPRT similarly catalyzes 5FU phosphorylation to 5FUMP, a key step in the 5FU activation pathway, and the rate-limiting step in mammalian cells due to the absence of a UPRT enzyme[71]. Miyagi *et al.* demonstrated that 5FU conversion in DU145 human prostate cancer cells is enhanced following bUPRT transduction, resulting in a 56.6-fold decrease in the 5FU IC_{50} value[72], while co-expression of bCD with bUPRT increases cell sensitivity to 5FC treatment 100-fold over bCD alone[73].

Expression of the bCD/bUPRT or yCD/yUPRT fusion constructs in C6 rat glioma cells enhanced cell sensitivity to 5FC relative to expression of bCD or yCD alone[27, 73-76]. Additionally, our lab demonstrated that fusion of bUPRT with the variant enzyme bCD_{1525} further increased C6 cell sensitivity to 5FC: the IC_{50} value for cells expressing bCD_{1525}/bUPRT was 10-fold lower than for those expressing bCD/UPRT[77]. We hypothesize that this sensitivity results from increased intracellular concentrations of the efficacious products 5FUMP and 5FdUMP as well as a decrease in 5FU catabolism by DPD. In order to study the effect of bUPRT expression on 5FU metabolism, we

developed and validated a novel HPLC method to detect and quantify FBAL concentrations (discussed in Chapter 3). Use of this HPLC method in conjunction with the method we developed to detect 5FC and four of its major anabolites (Chapter 2) will provide a more comprehensive understanding of the interplay between suicide enzymes and endogenous mammalian enzymes involved in 5FC metabolism. Additionally, if may indicate whether use of a fusion CD/UPRT enzyme will decrease 5FU concentrations, therefore making this a promising therapy for patients over-expressing or lacking functional DPD.

While pathway engineering enhances the cell's ability to convert prodrug to cytotoxic products, it may indirectly, and perhaps negatively, affect the bystander effect (BE). In the 5FC/bCD system, the main mechanism by which the BE occurs is through intercellular diffusion of 5FU[78]. However, upon expression of bCD/UPRT or bCD_{1525}/UPRT, we anticipate that bUPRT activity will decrease the cellular concentrations of diffusible 5FU, thereby shifting the BE mechanism away from 5FU diffusion. As noted above, a BE can result from phagocytosis of apoptotic vesicles or cells by neighboring cells or by movement of phosphorylated metabolites through gap junctions. Therefore, we evaluated the ability of cells expressing fusion enzymes to elicit a BE and assessed the contribution of apoptotic vesicles to bystander cell killing. Additionally, we quantified intercellular dye transfer in these cells as an measure of gap junction formation (Chapter 4).

In order to increase the likelihood of successful ablation gene therapy against cancers, many aspects of this therapy require improving. These areas include: 1) devising safe and efficient delivery strategies to limit suicide gene expression to

cancerous cells; 2) lowering or eliminating prodrug toxicity in patients; 3) creating suicide genes that do not themselves cause patient toxicity; and 4) evoking a strong BE to ensure complete tumor eradication. Our laboratory research currently focuses on areas 2-4. By using enzyme and pathway engineering, we generated several bCD and yCD enzymes that display an increased activity toward prodrug, increased thermostability, and enhance cell killing ability. Here we present a more in-depth examination of these enzymes in terms of prodrug metabolism and BE efficacy. We plan to utilize these data in future studies to improve enzymes in terms of prodrug metabolism and to engineer enzymes that elicit a more optimal BE or limit prodrug metabolism by endogenous mammalian enzymes.

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CHAPTER TWO

Development and Validation of a Rapid and Sensitive HPLC Method for the Quantification of 5-Fluorocytosine and Its Metabolites[1]

Abstract

To study the intracellular metabolism of the prodrug 5-fluorocytosine (5FC), we developed a novel reverse-phase high-performance liquid chromatography method to simultaneously detect 5FC and its four major anabolic metabolites: 5-fluorouracil, 5-fluorouridine, 5-fluorouridine-monophosphate, and 5-fluoro-2′deoxyuridine-5′-monophosphate. Separation of each compound was accomplished under isocratic conditions using a C_{18} column and mobile phase of formic acid:water (1:99 v/v). The method was validated for both accuracy and reproducibility in cell culture media. Additionally, metabolites were assessed for stability at ambient temperatures and following freeze-thaw cycles. Calibration curves were linear over a range of 1-200 µg/mL. Limit of quantification (LOQ) for four of the five compounds was 1 µg/mL in cell culture media (RSD<11%). This method was successfully used to monitor intracellular conversion of 5FC to its metabolic products over a twenty-four hour period.

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K.M. Serve was the main contributor to this work, including performing the validation of the method and cell culture experiments as well as preparing the manuscript for publication.

J.A. Yañez aided in determining suitable chromatographic conditions including UV detection wavelength and choice of mobile phase and internal standard.

C.M. Remsberg's contribution included technical assistance concerning standard curve preparation and validation protocols.

N.M. Davies and M.E. Black supervised this work, provided technical assistance, and scientific expertise.

Introduction

5-fluorouracil (5FU), a chemotherapeutic agent often administered in the treatment of breast, colorectal, and prostate cancers, is indiscriminately cytotoxic to cells, resulting in severe toxicity to patients upon systemic administration (Harris *et al.,* 1986). The nucleoside analog, 5-fluorocytosine (5FC), however, is an antifungal agent that is relatively non-toxic upon systemic administration. In bacteria, 5FC is deaminated by cytosine deaminase (CD) to produce 5FU. Since mammalian cells do not express cytosine deaminase enzymes, 5FU cytotoxicity can be preferentially localized within tumor sites by using 5FC as a prodrug for 5FU, coupled with tumor specific expression of the prodrug-converting enzyme bacterial CD (bCD), a process termed suicide gene therapy (Mullen *et al.*, 1992; Mullen *et al.*, 1994). 5FU is anabolized by cellular enzymes to 5-fluorouridine (5FURD), and subsequently to 5-fluorouridine-monophosphate (5FUMP). 5FUMP is further converted to 5-fluoro-2′deoxyuridine-5′-monophosphate (5FdUMP), which binds irreversibly to thymidylate synthase to inhibit both synthesis of thymidine triphosphate and subsequent synthesis of DNA (Mullen *et al.,* 1994; Huber *et al.,* 1994; Haberkorn *et al.,* 1996) (Figure 2.1).

Limiting factors of this therapy include high affinity of bCD for its natural substrate cytosine over the prodrug 5FC and the indirect conversion of 5FU to 5FUMP by endogenous enzymes. To address these issues, we are investigating the use of bCD variants with preferential affinity for 5FC as suicide enzymes (Fuchita *et al.,* 2009) as well as exploring the use of bCD in a fusion construct with the bacterial enzyme uracil phosphoribosyl transferase (bUPRT) as a means to directly convert 5FU to 5FUMP (Kawamura *et al,.* 2000; Miyagi *et al.,* 2003). Several investigators have reported that

expression of the fusion enzyme bCD/UPRT sensitizes cells to 5FC to a greater extent than expression of bCD alone (Tiraby *et al.,* 1998; Adachi *et al.,* 2000; Chung-Faye *et al.,* 2001; Erbs, *et al.,* 2000; Khatri *et al.,* 2006). This increased sensitivity presumably stems from enhancing enzyme activity toward prodrug and intermediate metabolites, thus resulting in an increased production of cytotoxic products. However, simultaneous detection and quantification of 5FC metabolite concentrations generated in cells expressing bCD or bCD/UPRT enzymes has not been previously reported. Understanding the metabolic activities of wild-type bCD and bCD/UPRT enzymes will aid in engineering new, more effective variants for use as suicide enzymes.

In order to assess 5FC metabolite production in mammalian cells, we developed a reverse-phase, isocratic high-performance liquid chromatography (HPLC) method to simultaneously detect 5FC and its four anabolic metabolites: 5FU, 5FURD, 5FUMP, and 5FdUMP. This method was used to detect intracellular conversion of 5FC in cultured cells expressing bCD or bCD/UPRT enzymes. Although HPLC methods have been used to detect 5FC, 5FU, and 5FdUMP (Huber *et al.,* 1994; Khatri *et al.,* 2006; Loos *et al.,* 1999; Toraño *et al.,* 2001; Wrightson *et al.,* 1995), to our knowledge, this is the first validated analytical method for the simultaneous detection of 5FC and its four major anabolic metabolites using HPLC.

Experimental Conditions

Chemicals and reagents

The compounds 5-fluorocytosine (5FC), 5-fluorouracil (5FU), 5-fluorouridine (5FURD), 5-fluoro-2′deoxyuridine-5′-monophospate (5FdUMP), and 5-chlorouracil were

purchased from Sigma-Aldrich (St. Louis, MO, USA). 5-fluorouridine-monophosphate (5FUMP) was purchased from Moravek Biochemicals (Brea, CA, USA). Powder forms of Dulbeco's Modified Eagle's Medium (DMEM) and Minimal Medium (MEM) were purchased from Invitrogen (Grand Island, NY, USA) and McCoy's Medium from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade water, acetonitrile, glacial acetic acid, and formic acid were purchased from JT Baker (Phillipsburg, NJ, USA). Media were prepared according to the manufacturer's instructions and the pH adjusted to 7.2. DMEM was supplemented with 5% FBS, MEM and McCoy's with 10% FBS. Blasticidin (4-6 µg/mL) was added to media for selection and maintenance of transfected cells. Reagents were prepared and filter sterilized using a 0.22 µm Steritop bottle top filter purchased from the Milipore Corporation™ (Billerica, MA, USA) prior to use in the HPLC system.

Chromatographic instrumentation and conditions

The HPLC system used was a Shimadzu HPLC (Kyoto, Japan) consisting of a LC-600 pump, a SIL-10AD*VP* auto injector, a SCL-10A system controller, and a SPD-10AV*VP* detector. Data collection and integration were performed using Shimadzu EZ Start 7.1.1SP1 software (Kyoto, Japan). Isocratic elution was performed at ambient temperature (25±1 ºC) on a Phenomenex Luna C_{18} (2) analytical column (250 mm x 4.6 mm, 5 µm particle size). The mobile phase consisted of formic acid and water (1:99, v/v), filtered and degassed prior to use. The flow rate was 1 mL/min, with ultraviolet detection at 285 nm, as determined by spectrophotometry. The injection volume was 200 µL.

Preparation of stock and standard solutions

Stock standard solutions of nucleotide analogs were prepared to a final concentration of 50 µg/mL by dissolving 2.5 mg powder in 50 mL HPLC grade water and shaking at 37 ºC for 30 min to facilitate dissolution. 5-chlorouracil (5ClU) was used as an internal standard (I.S.) and was prepared at a concentration of 100 µg/mL. 5FC, 5FU, 5FURD, and 5ClU were protected from light by wrapping in aluminum foil and stored at 4 °C for no longer than 4 months. Due to the unstable nature of phosphorylated compounds, 5FUMP and 5FdUMP were stored in 1.0 mL aliquots at -20 °C and discarded following three freeze-thaw cycles or four months, whichever came first.

Sample preparation in media

Standard solutions were prepared fresh prior to injection as follows. To each standard solution, 100 µg/mL I.S. and 100 µL medium were added. Samples were vortexed for 30 sec and dried until completion under a constant flow of compressed nitrogen gas. Samples were then reconstituted in mobile phase to final concentrations of 1, 5, 10, 50, 100, and 200 µg/mL. The injection volume was 200 µL.

Validation procedures

Linearity. A six-point standard curve at concentrations of 1, 5, 10, 50, 100, and 200 µg/mL was created for each of the five metabolites using peak-area-ratios (PAR) of standard compounds to internal standard, against metabolite concentrations using unweighted least squares linear regression analysis.

Accuracy, precision, and recovery. The within-run accuracy and precision of the replicate assays (n=5) were tested by using six different drug concentrations from 1-200 µg/mL. The between-run accuracy and precision of the assays (n=5) were estimated from the results of five replicate assays run on consecutive days. The precision was evaluated by the relative standard deviation (RSD). The accuracy was estimated based on the mean percentage error of measured concentration to the actual concentration (Shah *et al.,* 1991; Bansal and DeStafano*,* 2007).

The extraction efficiency (n=3) was determined by comparing the peak area ratio (PAR) of metabolites and I.S. to the PAR of corresponding concentrations of standard injected directly in the HPLC system without extraction.

Drug stability. The stability of 5FC and anabolic metabolites in DMEM over a 24 h period was determined for standards at the low and high metabolite concentrations of 5 and 50 µg/mL. A 200 µL sample was injected into the HPLC system at hour 0. The sample was then allowed to sit in the auto-injector at ambient temperature for 20, 22, or 24 h, at which time another 200 µL sample was injected. Freeze-thaw stability was evaluated at a metabolite concentration of 50 µg/mL (n=3). Standard samples were prepared and analyzed on the HPLC machinery. Samples were then subjected to three rounds of freeze-thaw cycles (frozen at -20 °C and thawed to 25±1 °C) and analyzed again. Stability at ambient temperatures and following freeze-thaw cycles were calculated by determining the percentage of metabolite peak area of the subsequent injections relative to peak area of the first injection.

In vitro evaluation of 5FC metabolites

C6 rat glioma cell lines (ATCC) were stably transfected with pcDNA vector carrying bCD or bCD/UPRT using FuGene 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA). Stably transfected cells were seeded (5×10^4) in 2 mL DMEM medium on 6-well plates (Corning Incorporation, Corning, NY, USA) and allowed to attach overnight. Cells were then incubated with approximately 325 µg/mL 5FC, or 2.5 mM, and incubated at 37 °C for up to five days. Reactions were stopped using 200 µL/well of 94:6 (v/v) acetonitrile/glacial acetic acid. Cells were collected with media using a sterile 25 cm cell scraper (Sarstedt, Newton, NC, USA) and counted on a hemacytometer. Cell membranes were lysed via three rounds of freeze-thaw and then centrifuged to remove cellular debris. Supernatant was removed and stored at -20 ºC. Prior to injection into the HPLC system, samples were thawed to room temperature and the internal standard 5-ClU (100 µg/mL) was added at 100 µL/1000 µL sample. Samples were vortexed, dried, and reconstituted in 600 µL of mobile phase. 200 µL of sample was injected into the HPLC system. To determine total levels (intracellular plus extracellular) of metabolites, peak area ratios were determined for each compound.

Results and Discussion

Validation procedures

Specificity. The HPLC retention times of the five metabolites and internal standard are: 5FC- 3.2 min, 5FU-5.7 min, 5FURD-12 min, 5FUMP-19 min, 5FdUMP-30 min, and I.S.-10 min. The developed method resulted in complete baseline separation of 5FC, metabolites, and the internal standard in the tested media with no apparent interference

between these compounds when eluting under the conditions outlined above. Since this method will be applied to cell culture samples, we needed to ensure that components of different media used in our lab did not interfere with peak detection. We observed no interfering peaks in blank DMEM, MEM, or McCoy's cell culture media samples with the compounds of interest (Figure 2.2). The peak corresponding with the 5FdUMP is broad compared to other compound peaks, indicating this compound is displaced from the column by the mobile phase less readily than the other compounds. Efforts to create a sharper peak by increasing the acidity of the mobile phase were unsuccessful. However, the broader peak of 5FdUMP did not adversely affect peak integration and analysis.

Linearity and limits of quantification (LOQ). The standard curves for the metabolites 5FC, 5FU, 5FURD, 5FUMP, and 5FdUMP were linear over the investigated range ($R^2 \geq 0.998$) as determined by comparing PAR values of metabolites to the internal standard. The y-intercept for mean regression lines from the validation runs were regressed and the slopes were described by 5FC (µg/mL)=0.0076±0.0022, 5FU (µg/mL)=0.0024±0.0002, 5FURD (µg/mL)=0.003±0.0001, 5FUMP (µg/mL)=0.0026±0.0007, and 5FdUMP (µg/mL)=0.0008±0.0001.

The LOQ of this assay was 1 µg/mL for all compounds except 5FdUMP, which had a higher LOQ of 5 µg/mL. 5FC displayed an upper LOQ of 200 µg/mL, above which the column became saturated and quantification was not accurate. The concentration of quality control samples calculated using linear regression was within the international harmonization acceptance criteria (Shah *et al.,* 1991*).* (Table 1).

Accuracy, precision, and recovery. The within- and between-run precision (RSD) calculated during replicate assays was <10.4% in all compounds over the standard solution concentrations. The intra- and inter-run bias assessed during replicate assays for 5FC varied between -1.21% and 10.15%, for 5FU between -8.3% and 11.5%, for 5FURD between -2.0% and 11.0%, for 5FUMP between -8.0% and 0.9%, and for 5FdUMP between -10.7% and 4.9%. These data indicate the developed HPLC method is accurate and precise at all concentrations examined. The mean extraction efficencies ranged from 83% to 115% for all compounds, suggesting that there was a negligible loss of compound during the drying process and that recovery is comparable between compounds.

Stability. Stability at ambient temperature over 22 h was excellent for all compounds with a mean recovery for these compounds of 101% at 5 µg/mL and 97% at 50 µg/mL. However, by 24 h, the mean recovery decreased to 82% at both 5 and 50 µg/mL, suggesting that after 22 h, rate of compound degradation increases. However, the rate of degradation for the internal standard closely follows that of the compounds, so after 24 h at room temperature, the mean PAR value of the compounds did not change compared to the mean PAR value at 0 h. Compound stability following three freeze-thaw cycles was excellent (mean recovery range of 101-119% at 50 µg/mL) for all compounds, including the internal standard.

Detection of intracellular 5FC conversion by bCD enzymes

This method was applied to mammalian cell culture samples to examine intracellular conversion of 5FC. A representative chromatogram of the metabolites detected in cell lysates confirms the specificity of this method in the presence of cellular protein (Figure 2.3A). Retention times shifted slightly for later compounds (5FURD, 5FUMP, 5FdUMP, and IS) likely due to the increased pH of the samples resulting from addition of the stop solution. However, the position of the metabolite peaks relative to the I.S. peak remained constant, allowing for compound identification.

Following a 24 h incubation with 5FC, we observed slightly higher 5FU and slightly lower 5FUMP levels in lysates of cells expressing bCD compared to those expressing bCD/UPRT (Figure 2.3B). Additionally, at this time point, 5FdUMP levels are more than 2-fold higher in lysates of bCD/UPRT expressing cells compared to bCD, supporting the theory that the presence of bUPRT enhances the production of cytotoxic metabolites. Theoretically, cytotoxic metabolites are produced at greater levels due to bUPRT directly converting 5FU to 5FUMP, thus reducing formation of the intermediate 5FURD. This study supports this theory as we observed an approximately 5-fold decrease in 5FURD levels in the lysates of cells expressing bCD/UPRT relative to bCD (Figure 2.3B).

Taken as a whole, these data illustrate that upon bCD expression, 5FC is metabolized to 5FU, 5FURD, 5FdUMP, and to a lesser extent 5FUMP. However, overall production of 5FdUMP was increased dramatically by the addition of bUPRT into the system, likely due to the decrease in 5FURD production. While bCD/UPRT expression results in greater production of 5FdUMP than bCD alone, *in vitro* cytotoxicity

assays indicate these quantities are not sufficient to achieve cell killing levels for effective tumor ablation. This HPLC method will allow additional characterization of optimized enzymes currently under construction in our laboratory, as well as to facilitate a deeper understanding for the chemical basis of differences in drug responses between various cell types. The overall goal of this project is to increase the activity of bCD and bUPRT toward 5FC and 5FU, respectively, thus increasing production of 5FdUMP to achieve more effective cancer cell killing.

Conclusions

In summary, we developed and validated an HPLC method for simultaneous detection of 5FC and four of its major anabolic metabolites (5FU, 5FURD, 5FUMP, and 5FdUMP). To our knowledge, no previously published method has reported the simultaneous detection of these five compounds. The method is sensitive over a concentration range of 1-200 μg/mL and is accurate and reproducible. Application of this method was shown by detection of intracellular 5FC conversion to metabolic products in cells stably expressing the bCD or bCD/UPRT enzyme.

Ongoing studies in our laboratory focus on understanding *in vitro* bCD-mediated metabolism of 5FC in order to aid in engineering enzyme variants that are more efficient at converting prodrug to cytotoxic product. The development of an HPLC method that simultaneously detects 5FC and its anabolic metabolites will allow us to quantify and better understand the activity of these variants when expressed in mammalian cells.

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Table 2.1. Standard curve parameter summary and mean back-calculated calibration curve concentrations (µg/mL) assessed within (upper) and between (lower) days (n=5). Standard deviations averages range between 0.9 and 3.2.

Conc. (pg/mL)	5FC	RSD (%)	Bias (%)	5FU	RSD (%)	Bias (%)	5FURD	RSD (%)	Bias (%)	5FUMP	RSD (%)	Bias (%)	5FdUMP	RSD (%)	Bias (%)
1	1.0	6.8	-0.02	1.0	9.3	-4.8	1.0	6.4	-2.0	1.0	4.2	-2.8	*ND	*ND	*ND
5	5.0	10.5	-1.1	5.3	10.2	6.6	5.6	9.3	11.0	5.0	6.5	0.2	5.1	8.8	2.0
10	10.3	7.5	2.6	10.3	9.7	3.1	10.0	8.2	0.5	9.8	3.2	-1.9	9.8	7.5	-2.0
50	50.6	6.0	1.2	49.2	8.2	-1.6	51.6	4.5	3.2	50.3	8.1	0.6	51.6	9.9	3.1
100	101.3	6.5	1.3	98.1	5.9	-1.9	98.0	2.2	-2.0	99.5	1.5	-0.5	100.0	5.3	-0.1
200	199.7	1.4	-0.1	202.4	1.4	1.2	200.7	1.1	0.3	199.1	1.5	-0.5	197.1	4.2	-1.5

Conc. (pg/mL)	5FC	RSD (%)	Bias (%)	5FU	RSD (%)	Bias (%)	5FURD	RSD (%)	Bias (%)	5FUMP	RSD (%)	Bias (%)	5FdUMP	RSD (%)	Bias (%)
1	1.1	10.6	10.2	1.0	9.6	-4.7	1.0	7.0	-1.1	1.0	1.9	-1.2	*ND	*ND	*ND
5	5.5	6.6	9.9	4.6	9.3	-8.3	5.4	4.8	7.0	4.6	10.7	-8.0	5.2	0	4.9
10	10.6	4.1	6.1	11.5	2.0	11.5	10.1	9.9	1.2	10.1	3.5	0.9	9.0	5.1	-10.4
50	51.1	1.3	2.3	48.8	4.8	-2.4	50.7	6.3	1.3	47.1	0.9	-5.9	52.3	2.7	4.6
100	107.5	3.2	7.5	99.3	6.4	0.7	101.6	5.0	1.6	99.4	1.7	0.6	89.3	5.2	10.7
200	197.6	0.4	-1.2	202.2	1.0	1.1	201.3	0.7	0.6	198.4	1.5	-0.8	188.2	1.2	-5.9

Figure Legends

Figure 2.1. 5FC activation pathway in mammalian cells transfected with the bacterial genes encoding CD and UPRT. Bacterial enzymes are underlined and bolded. CD, cytosine deaminase; UPRT, uracil phosphoribosyltransferase; TS, thymidylate synthase; 5FC, 5-fluorocytosine; 5FU, 5-fluorouracil; (5F)UMP, (5-fluoro) uridine monophosphate; (5F)dUMP, (5-fluoro) deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate, dTDP, deoxythymidine diphosphate, dTTP, deoxythymidine triphosphate.

Figure 2.2. Representative chromatograms of standard curves. Curves were prepared in DMEM (A), MEM (D), and McCoy's (G) media and contain 5FC and metabolites at 50 µg/ml and I.S. at 100 µg/mL. Chromatograms representing media blanks are shown of DMEM (B), MEM (E), and McCoy's (H) media. Overlays of chromatograms depicting metabolites (—) and media blanks (····) illustrate no interfering peaks are present between metabolites, internal standard, or media for DMEM (C), MEM (F), and McCoy's (I). Metabolite separation was achieved isocratically using a reverse-phase HPLC method. UV detection of the compounds was attained at 285 nm. The mobile phase consisted of formic acid and HPLC water (1:99, v/v) with a flow rate of 1 mL/min.

Figure 2.3. (A) Representative chromatogram of 5FC and metabolites detected in lysates of C6 cells. (B) Concentrations (µg/mL) of the metabolites 5FU, 5FURD, 5FUMP, and 5FdUMP detected in lysates of C6 cells expressing bCD or bCD/UPRT.

Metabolite levels following a 24 h incubation with 5FC (325 µg/mL) are shown as µg/mL.

Figure 2.1

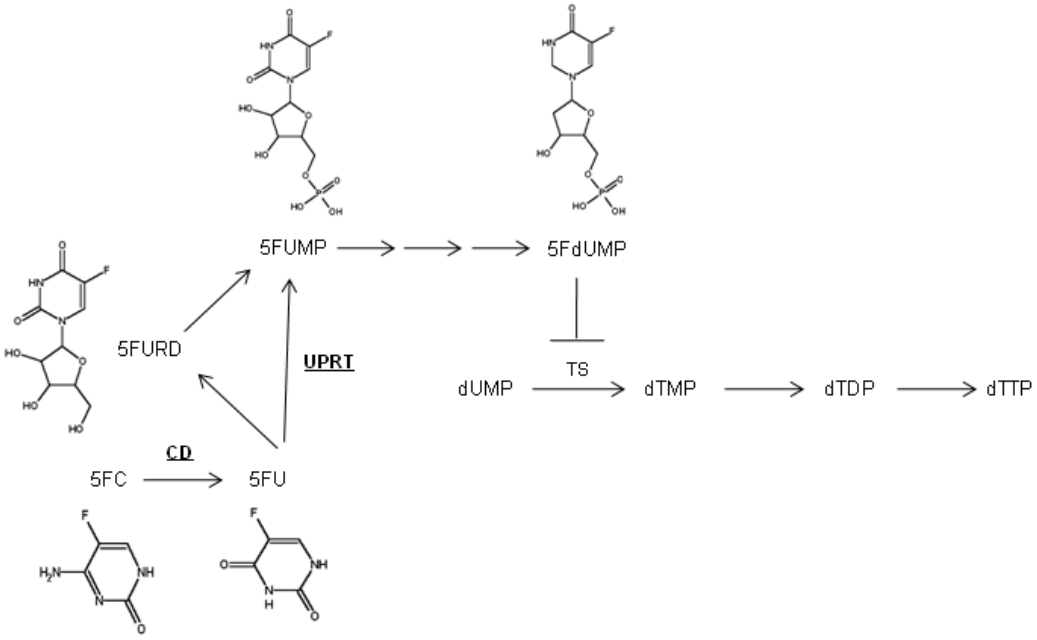

Figure 2.2

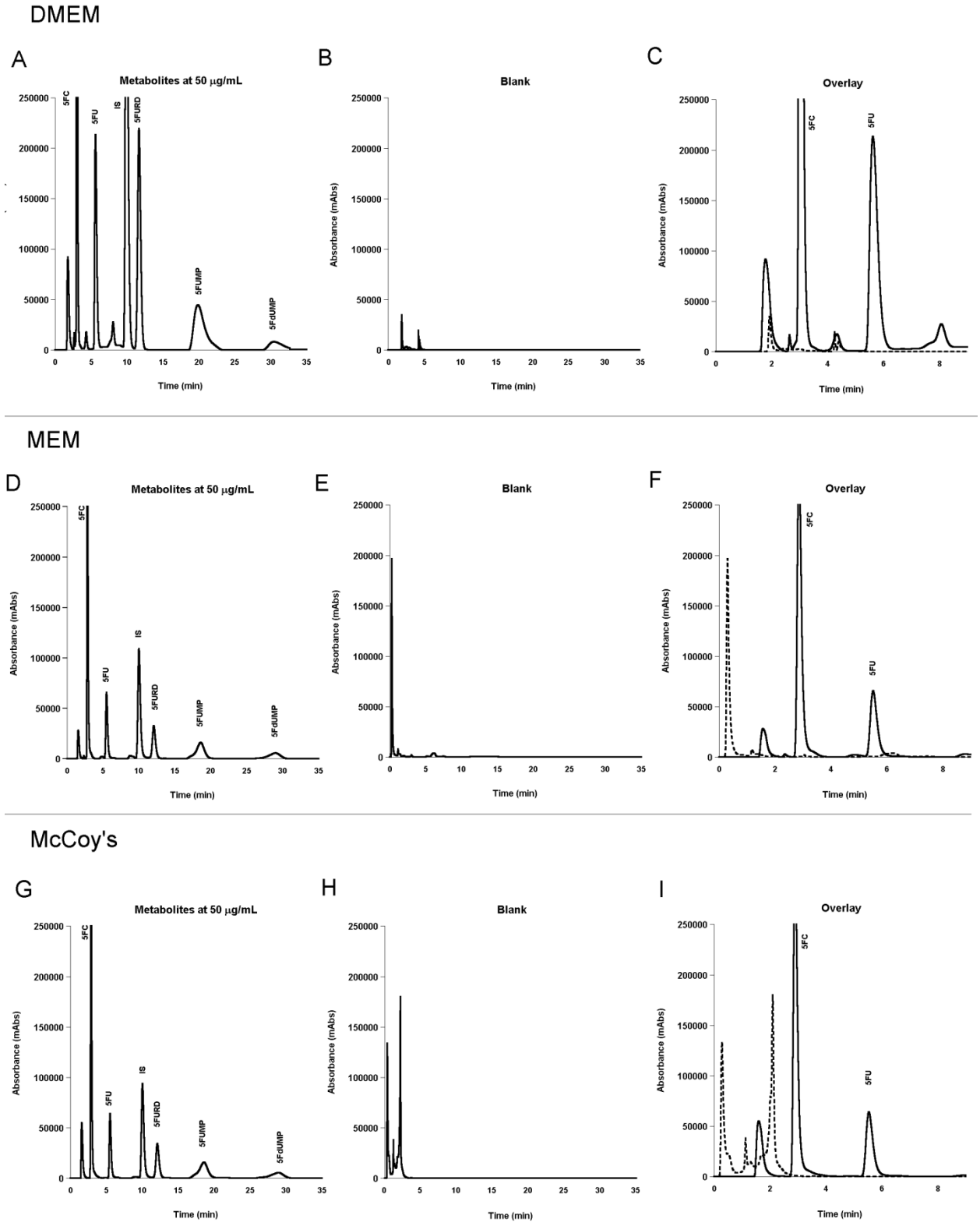
DMEM
A
Metabolites at 50 µg/mL
5FC
5FU
IS
5FURD
5FUMP
5FdUMP
Absorbance (mAbs)
Time (min)
B
Blank
C
Overlay
MEM
D
E
F
McCoy's
G
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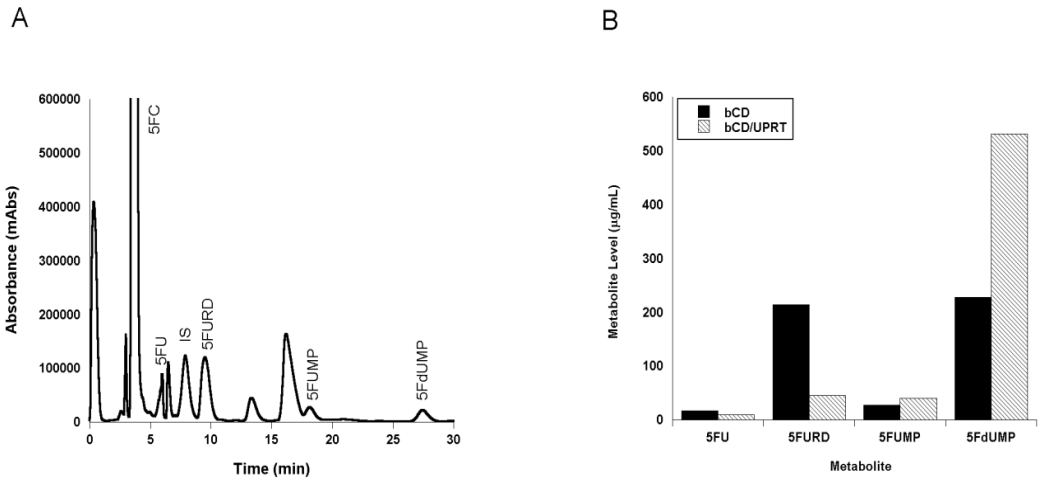
Figure 2.3


A
600000
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Absorbance (mAbs)
5FC
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5FURD
5FUMP
5FdUMP
0
5
10
15
20
25
30
Time (min)
B
600
500
400
300
200
100
0
Metabolite Level (µg/mL)
bCD
bCD/UPRT
5FU
5FURD
5FUMP
5FdUMP
Metabolite

CHAPTER THREE

Validation of an Isocratic HPLC Method to Detect 2-Fluoro-β-Alanine for the Analysis of Dihydropyrimidine Dehydrogenase Activity[2]

Abstract

The efficacy of the chemotherapeutic drug 5′-fluorouracil is reduced by catabolism to 2′-fluoro-β-alanine (FBAL), a reaction catalyzed by dihydropyrimidine dehydrogenase (DPD). To study *in vitro* DPD activity, we developed and validated an isocratic, reverse-phase HPLC method to detect and quantify FBAL without using multiple columns or radiolabeled substrates. Pre-column derivatization of FBAL was performed using *o*-phthalaldehyde in the presence of two sulfur donors, ethanthiol or β-mercaptoethanol, and the resulting products assayed. Calibration curves were linear over a range of 10-200μg/ml and the method was successfully applied to the examination of DPD activity in cultured cells.

[2] This manuscript was submitted to the *Journal of Chromatography B* and is formatted in accordance with the journal requirements. It was submitted as: K.M. Serve, J.L. Darnell, J.K. Takemoto, N.M. Davies, and M.E. Black.

K.M. Serve was the main contributor to this work, including performing the method validation and cell culture experiments as well as preparing the manuscript for publication.

J.L. Darnell aided in determining a suitable mobile phase and internal standard. Additionally, this author aided in preparing the standard curves and performed the stability assays.

J.K. Takemoto performed the LC/MS assays and provided technical assistance.

N.M. Davies and M.E. Black supervised this work, provided technical assistance, and scientific expertise.

1. Introduction

The chemotherapeutic drug 5′-fluorouracil (5FU) is commonly used to treat prostate, colorectal, breast, and pancreatic cancers. However, systemic 5FU administration can cause severe side effects in patients, including gastrointestinal distress, myelosuppression, and cardiotoxicity [1,2]. Alternatively, administration of a nontoxic prodrug of 5FU coupled with tumor-specific expression of a prodrug-activating enzyme may ameliorate toxicity; this treatment strategy is termed suicide gene therapy. The enzyme/prodrug system bacterial cytosine deaminase/5′-fluorocytosine (bCD/5FC) has been widely studied for use in suicide gene therapy. Following transfection with the bCD enzyme, tumor cells become 5FC sensitive while CD deficient cells remain relatively 5FC resistant [3,4].

The effectiveness of the bCD/5FC system for suicide gene therapy is reduced by the expression of the endogenous mammalian enzyme dihydropyrimidine dehydrogenase (DPD; EC 1.3.1.2). DPD normally acts in the uracil catabolism pathway but also catalyzes the catabolism of 5FU to dihydrofluorouracil (DHFU). DHFU is further metabolized to 2-fluoro-β-alanine (FBAL), a compound readily excreted in patient urine (Figure 3.1). When patients express DPD at normal ranges, >80% of systemically administered 5FU is excreted as FBAL while over-expression of DPD is associated with reduced 5FU serum exposure, half-life and efficacy, and increased patient mortality [5-7]. We predict that DPD-mediated catabolism of 5FU will be reduced by expression of another enzyme, bacterial uracil phosphoribosyltransferase (bUPRT), with bCD in suicide gene therapy. bUPRT mediates 5FU anabolism to 5-fluorouridine monophosphate (5FUMP), which is further converted to cytotoxic products that interfere

with DNA and/or RNA synthesis, resulting in cell death [8,9]. In theory, expression of bUPRT either concomitantly or in a fusion with bCD will presumably lower the intracellular concentrations of 5FU available as substrate for DPD, thus decreasing formation of FBAL (Figure 3.1).

To study the role DPD plays in 5FC metabolism, we developed a simple, reverse phase high-performance liquid chromatography (HPLC) method to resolve *o*-phthalaldehyde (OPA) derivatives of FBAL from the structurally similar endogenous amino acids L-alanine and β-alanine. Unlike other previously reported methods describing the separation of these compounds, this method is isocratic, utilizes a single column, and does not rely on radiolabeled substrates to achieve sensitive and reliable FBAL detection [10-15]. In other studies, the stability of OPA derivatives of alanine and β-alanine were determined to be more stable at room temperature when the derivatization reaction was carried out using ethanthiol (ET) as the sulfur donor as opposed to the more commonly used β-mercaptoethanol (MCE) [16,17]. However, there have been no reports of similar stability studies performed on OPA-derivatives of FBAL. Therefore, we compared the stability of FBAL when the OPA derivatization reaction was carried out in the presence of ET or MCE. Herein the establishment of a novel HPLC method for FBAL detection and comparative results of two different derivatization methods are described.

2. Materials and methods

2.1 Chemicals and reagents

The compound DL-2-fluoro-β-alanine (FBAL) was purchased from Indofine (Hillsborough, NJ, USA) and 5-fluorouracil (5FU), L-alanine, MCE, and OPA were

purchased from Sigma-Aldrich (St. Louis, MO, USA). ET and β-alanine were purchased from Alfa Aesar (Ward Hill, MA, USA). Pterostilbene was a gift from the Sabinsa Corporation (Piscataway, NJ, USA). Potassium borate was purchased from Pfaltz & Bauer, Inc. (Waterbury, CT, USA). HPLC grade water, acetonitrile, methanol, and phosphoric acid were purchased from JT Baker (Phillipsburg, NJ, USA). McCoy's medium was purchased from Sigma-Aldrich (St. Louis, MO, USA), prepared according to the manufacturer's instructions, supplemented with 10% FBS, adjusted to a pH of 7.2, and filter sterilized using a 0.22 µm Steritop® bottle top filter (Milipore Corporation, Billerica, MA, USA).

2.2 Preparation of stock and standard solutions

Stock solutions of alanine, β-alanine, and FBAL were prepared in HPLC-grade methanol to a final concentration of 200 µg/ml. Pterostilbene, used as an internal standard (I.S.), was prepared at a concentration of 100 µg/ml in HPLC-grade methanol. These solutions were wrapped in aluminum foil to protect from light and stored at -20 °C. OPA stock solution (designated methanolic OPA) was prepared in HPLC grade methanol to a final concentration of 26 M prior to derivatization reactions, as previously described[15-17].

2.3 Derivatization procedure

The buffer solution was prepared by dissolving potassium borate crystals in HPLC-grade water to a final concentration of 0.6 M, pH=10. Derivatization reactions were carried out as follows:

a) 15 µl MCE was added to 2.5 ml buffer solution and mixed with 0.5 ml methanolic OPA. The OPA-MCE reagent solution was added to amino acid solutions at 50% (v/v).

b) 52 µl ET was added to 2 ml buffer solution and mixed with 0.63 ml methanolic OPA and brought to a final volume of 10 ml with methanol. The OPA-ET reagent solution was added to amino acid solutions at 80% (v/v).

The derivatizing agents were stored at room temperature and discarded after 48 hours.

2.4 Instrumentation and chromatographic conditions

The HPLC system used was a Shimadzu HPLC (Kyoto, Japan) consisting of a LC-20AD pump, SIL-10AD*VP* auto injector, SCL-10A system controller, and SPD-10AV*VP* detector. Data collection and integration were performed using Shimadzu EZ Start 7.1.1SP1 software (Kyoto, Japan). The OPA-derivatives of L-alanine, β-alanine, and FBAL were separated under isocratic conditions at ambient temperature (25±1ºC) at a flow rate of 0.6 ml/min on a Phenomenex Luna® C_{18} (2) analytical column (250 mm x 4.6 mm, 5 µm particle size). The column was outfitted with a Phenomenex Security Guard Analytical Guard Cartridge System®. Mobile phase consisted of 65% MeOH, 15% ACN, 19.9% H_2O, and 0.1% H_3PO_4 (v/v) and was filtered and degassed prior to use. Derivatized products were detected at the ultraviolet wavelength of 334 nm, and were identified by their retention times as compared to commercial standards.

The liquid chromatography-mass spectrometry-electrospray ionization (LC-MS-ESI) system employed was a Shimadzu LCMS-2010 EV system (Kyoto, Japan) with the LC portion consisting of two LC-10AD pumps, a SIL-10AF *VP* auto injector, a SPD-10A

VP UV detector, and a SCL-10A *VP* system controller. Data analyses were performed using Shimadzu LC-MS LabSolutions Version 3 software (Kyoto, Japan). The chromatographic methods were identical to those employed in the HPLC methodology except that H_3PO_4 in the mobile phase was replaced by formic acid at 0.1% total volume. The mass spectrometer conditions consisted of a curved desolvation line (CDL) and a heat block temperature of 200 °C. The CDL, interface, and detector voltages were -10.0 V, 4.5 kV, and 1.5 kV, respectively. Vacuum was maintained by an Edwards® E2M30 rotary vacuum pump (Edwards, UK). Liquid nitrogen was used as a source of nebulizer gas (1.5 L/min).

2.5 Validation criteria

2.5.1 Sample preparation in McCoy's medium

Standard solutions were prepared fresh from stock solutions. The I.S. pterostilbene (100 µg/ml) and 100 µl McCoy's medium were added to each sample and vortexed for 30 sec. The solutions were dried until completion under a constant flow of compressed nitrogen gas. The samples were reconstituted in 100 µl mobile phase and derivatized as described in Section 2.3.

2.5.2 Linearity

A six-point standard curve of FBAL was generated by diluting stock solution of FBAL in HPLC grade water to final concentrations of 5, 10, 25, 50, 100, and 200 µg/ml. I.S. (100 µl) was added to each sample. The peak-area-ratios (PAR) of FBAL to I.S.

were plotted against theoretical FBAL concentrations and linearity was calculated using unweighted least squares linear regression analysis.

2.5.3. Accuracy, precision, and recovery

The intra-day (n=8) and inter-day (n=6) accuracy and precision of replicate assays were tested by using these six-point standard curves of FBAL as described above. Precision was evaluated by the relative standard deviation (RSD). Accuracy was estimated based on the mean percentage error of measured concentration to the actual concentration (Bias).

2.5.4. Stability of FBAL and derivatives

Low and high concentrations (10 and 200 µg/ml, respectively) of FBAL derivatized with OPA-ET or OPA-MCE were assessed for stability following a 24 h incubation at ambient temperature (n=3). FBAL and I.S. standards were prepared in McCoy's medium, dried, and reconstituted in the mobile phase. Derivatization reactions were carried out as described in Section 2.3 and 100 µl of sample was injected at 0 h. The solution remained at ambient temperature in the auto-injector followed by subsequent injections at various time points up to 24 h. Freeze-thaw stability was evaluated for FBAL at concentrations of 10 and 200 µg/ml (n=3) according to guidelines for method validation [18]. Following the addition of I.S. and McCoy's medium (100 µl each), samples were dried and reconstituted in 200 µl mobile phase. An aliquot (100 µl) was derivatized with OPA-ET and injected. A second aliquot (100 µl) was subjected to

three freeze-thaw cycles (-80 °C) followed by derivatization and injection into the HPLC system.

2.6 Mammalian cells

HCT116 human colorectal cancer cell lines (ATCC) were maintained at 37 °C and 5% CO_2 in McCoy's Medium supplemented with 10% Fetal Bovine Serum. Cells were stably transfected with the mammalian expression vector pcDNA 6 carrying the genes encoding the enzymes bCD or bCD/UPRT. Cells expressing empty pcDNA vector were used as controls. Transfection was performed using FuGene 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to manufacturers' recommendations. Positive selection and maintenance of transfectants was accomplished by culturing cells in medium supplemented with 6 μg/ml blasticidin. Protein expression was confirmed by immunoblot analysis.

2.7 Evaluation of DPD expression and activity

Expression of DPD in HCT116 human colorectal cancer cells (ATCC) was analyzed using immunoblot analysis. Cells ($1x10^5$ cells/μl) were lysed in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Proteins were separated on a 12% SDS-PAGE gel and transferred to a 0.2 μm nitrocellulose membrane (BioRad, Hercules, CA, USA). The primary antibody to DPD (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied at a 1:300 dilution in TTBS [Tris-Buffered Saline (1 M Tris, pH 7.5, 5 M NaCl) plus 0.05% Triton X] and incubated overnight followed by application of the secondary goat anti-rabbit antibody (BioRad, Hercules, CA, USA) at a 1:2000

dilution in TTBS. Reactive bands were visualized using colorimetric reagents (BioRad, Hercules, CA, USA) and identified by protein size as compared to a standard ladder (BioRad, Hercules, CA, USA). Commercially available whole cell lysates of HL-60 (human promyelocytic leukemia cells, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as a positive control for DPD expression, as suggested by the manufacturer.

Stably transfected HCT116 cells were seeded in 6-well plates at $2x10^5$ cells/well and incubated with 645 μg/ml 5FC, final concentration in 2 ml McCoy's, at 37 °C. After 24 h incubation, 200 μl of acetonitrile:acetic acid (94:6, v/v) was added to each well. Cells were lysed via freeze-thaw and debris removed by centrifugation. I.S. (100 μg/ml) was added and samples were dried, reconstituted in mobile phase, and derivatized with OPA-ET as described in Section 2.3. Following filtration through a 0.2 μm membrane syringe filter (Pall Corporation, Ann Arbor, MI, USA), 100 μl of the supernatant was injected into the HPLC system.

3. Results and discussion

3.1. Derivatization, stability, and specificity

Derivatization reactions of FBAL were carried out as described in Section 2.3 and the identity of the products was confirmed using commercially purchased standards and by LC-MS analysis. The expected m/z ratios of the OPA-ET derivatives in the electropositive ion mode were calculated as formula weight of the compounds (FBAL, 107.1; L-alanine and β-alanine, 89.1; pterostilbene, 256.3) plus OPA-ET (180.3). The schematic reaction sequence for the OPA-ET derivatized FBAL is outlined in Figure 3.2. The stability of the OPA FBAL derivatives (10 and 200 μg/ml) was analyzed at ambient

temperature. Mean recovery (n=3) of OPA-ET derivatives was >88%±3 up to 24 h at both concentrations examined. Recovery of OPA-MCE derivatives was poor as the compound was not detectable after 3 h. Similar results were obtained in stability analyses of OPA-ET and OPA-MCE derivatives of L-alanine and β-alanine [16,17].

The developed HPLC method resulted in the following retention times of OPA-ET derivatives: FBAL- 10 min and I.S.- 7.8 min. When the analyte standards were run in McCoy's medium, no interfering peak was noted for the FBAL derivative. A minor peak is observed at the elution time of the I.S. but this did not interfere with quantification at the I.S. concentrations used (Figure 3.3). The peaks corresponding with the OPA-ET derivatives of L-alanine and β-alanine eluted at 9 min. As the focus of this study was the identification and quantification of FBAL, resolution of the L-alanine and β-alanine peaks was not pursued.

3.2. Linearity and lower limits of quantification (LLOQ) and detection (LLOD)

Standard curves of FBAL were linear over a range of 10-200 μg/ml (R^2≥0.991) as determined by comparing PAR values of FBAL to the I.S. A typical mean regression curve is described by FBAL (μg/ml)=0.005±0.001*x*+0.1±0.05. The LLOQ and LLOD of this method was 10 μg/ml.

3.3. Accuracy, precision, and recovery

Accuracy and precision of the method were evaluated over a range of FBAL concentrations from 10-200 μg/ml. The RSD and bias for intra-day (n=8) and inter-day

(n=6) replicates (Table 3.1) is within the accepted validation criteria as set forth by the Food and Drug Administration Guidance Documents [18].

3.4. Stability of FBAL

Stability of FBAL (n=3) following three freeze-thaw cycles was excellent with a mean recovery of 103%±19% at 10 µg/ml and 107%±24% at 200 µg/ml.

3.5. Evaluation of DPD expression and activity

We applied the HPLC method to assess *in vitro* DPD metabolic activity in HCT116 cells as described in Section 2.6. DPD expression in two cell lines was confirmed by immunoblot analysis (Figure 3.4A). A protein band corresponding to DPD was observed at 90 kDa in HCT116 cells as well as HL60 cells used as a positive control. DPD activity in cells was estimated by examining intracellular conversion of 5FC to FBAL in HCT116 cells expressing the suicide enzyme bCD or the fusion enzyme bCD/UPRT. Samples were prepared as described in Section 2.6 (n=3). FBAL was detected in cells expressing both bCD or bCD/UPRT 24 h post-dose, demonstrating that this HPLC method is suitable for *in vitro* detection of FBAL (Figure 3.4B). FBAL concentrations in cells expressing bCD/UPRT were slightly lower as compared to bCD expressing cells, suggesting that expression of bUPRT may partially limit DPD-mediated catabolism of 5FU, at least initially. Little FBAL (11 µg/ml) was detected in control cells expressing empty vector. Since these cells lack an enzyme to metabolize 5FC, the levels detected were most likely a result of spontaneous deamination of 5FC to 5FU followed by conversion of the 5FU to FBAL by DPD. However, since the FBAL

levels detected in control cells were significantly lower than those detected in enzyme-expressing cells, they do not interfere with accurate FBAL detection or quantification in our experiments.

4. Conclusions

In summary, we developed a reverse-phase, isocratic HPLC method to rapidly (<12 min) separate and quantify FBAL. Detection of OPA-ET derivatives of FBAL was accurate and precise over a concentration range of 10-200 μg/ml and FBAL is stable following freeze-thaw cycles. Additionally, stability of OPA-ET derivatives was excellent at room temperature for up to 24 h whereas OPA-MCE derivatives quickly degraded. To our knowledge, this is the first report comparing stability of FBAL when derivatized by OPA in the presence two different sulfur donors. This method was successfully utilized to examine *in vitro* activity of the enzyme DPD. Unlike previously reported methods for examining DPD activity, FBAL detection was achieved without use of multiple columns, mobile phase gradients, or radio-labeled substrates, making this a simple, rapid, and convenient assay for *in vitro* applications of suicide gene therapy. This method can also be used in conjunction with our previously published method [19] for quantification of 5FC and it four major anabolic metabolites to enable a comprehensive analysis of *in vitro* 5FC metabolism. Additionally, this method may be further refined for *in vivo* pre-clinical and clinical studies or for use as a diagnostic tool for assessing *in vivo* DPD activity.

5. References

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6. Figure legends and tables

Figure 3.1: 5FC activation pathway in mammalian cells transfected with bacterial genes (denoted by *). bCD, bacterial cytosine deaminase; bUPRT, bacterial uracil phosphoribosyltransferase; DPD, dihydropyrimidine dehydrogenase; 5FC, 5-fluorocytosine; 5FU, 5-fluorouracil; 5FUMP, 5-flurouridine monophosphate; DHFU, dihydrofluorouracil; FBAL, 2-fluoro-β-alanine.

Figure 3.2: Proposed reaction mechanism of FBAL derivatization. The product of reaction 1 is the derivatizing agent OPA-ET, and of reaction 2 is OPA-ET-FBAL. Molecular weights values are noted in parentheses below each compound.

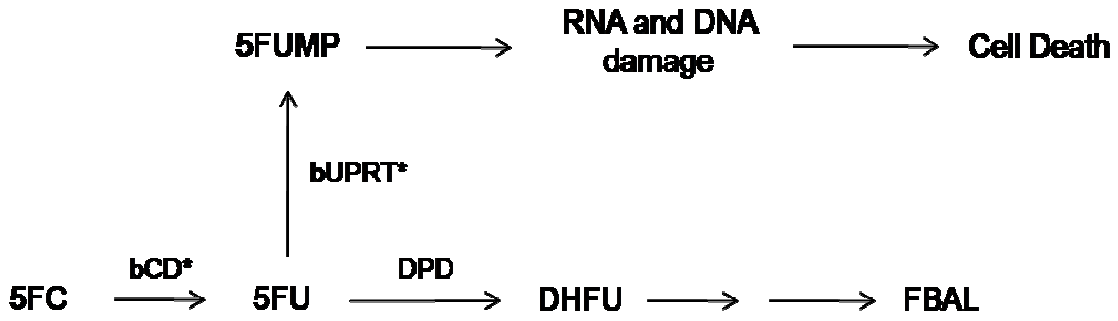
Figure 3.3: Representative chromatogram illustrating separation of OPA-ET derivatives of FBAL and I.S. No interfering peaks between FBAL (solid line) and McCoy's medium (dashed line) are observed at a 0.6 ml/min flow rate. A minor peak eluted with the I.S. but did not interfere with quantification at the I.S. concentrations used.

Figure 3.4: Evaluation of DPD expression and activity in mammalian cells. A) Representative immunoblot illustrating expression of the DPD protein (~90 kDa) in HCT116 and HL-60 cells. B) Concentrations (μg/ml) of FBAL detected in lysates of HCT116 cells expressing bCD or bCD/UPRT enzymes and control cells. Data illustrate metabolite levels following 24 h incubation with 645 μg/ml 5FC in medium.

Table 3.1: Standard curve parameter summary and mean back-calculated calibration curve concentrations and standard deviations (S.D.) for intra-day (n=8) and inter-day (n=6) runs.

	Within			Between		
FBAL (µg/ml)	**Mean±S.D.**	**RSD (%)**	**Bias (%)**	**Mean±S.D.**	**RSD (%)**	**Bias (%)**
10	11.6±2.1	18.1	15.6	11.0±1.7	15.3	9.7
25	23.9±2.6	10.9	-4.5	22.1±1.3	5.7	-11.7
50	49.5±6.6	13.3	-1.1	48.6±4.7	9.6	-2.8
100	104.0±9.3	8.9	4.0	101.7±4.2	4.2	1.7
200	198.8±4.3	2.2	-0.6	199.0±1.7	0.9	-0.5

Figure 3.1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RNA and DNA damage
Cell Death
bUPRT*
bCD*
DPD
5FC
5FU
DHFU
FBAL

Figure 3.2

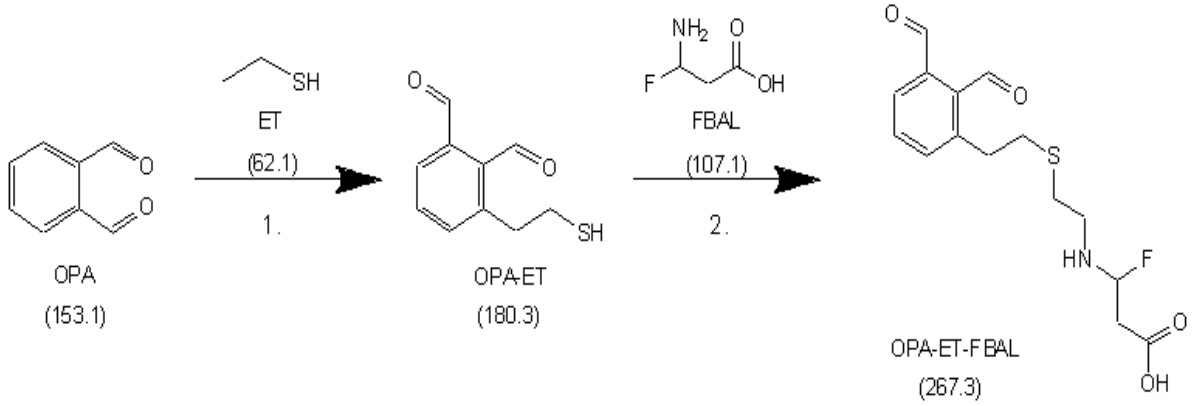

O
O
OPA
(153.1)
SH
ET
(62.1)
1.
O
O
SH
OPA-ET
(180.3)
NH2
O
F
OH
FBAL
(107.1)
2.
O
O
S
HN
F
O
OH
OPA-ET-FBAL
(267.3)

Figure 3.3

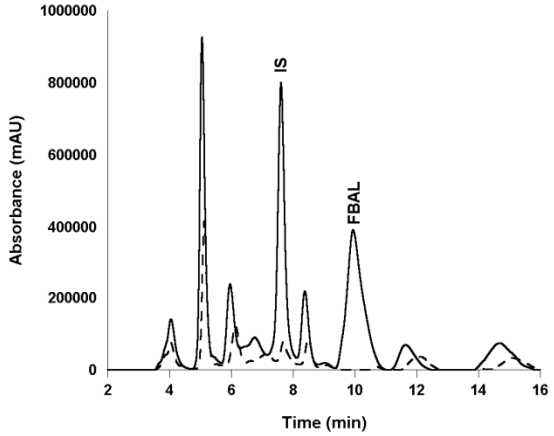
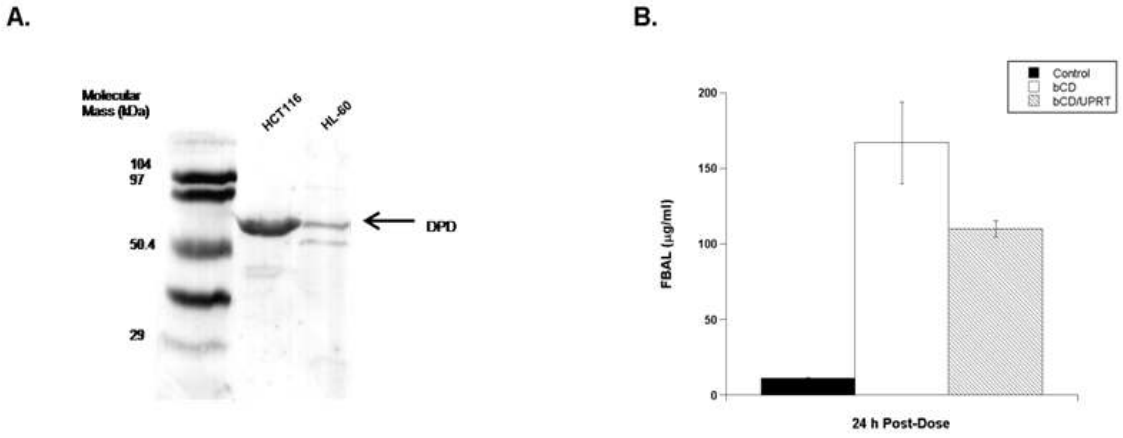

1000000
800000
600000
400000
200000
0
Absorbance (mAU)
IS
FBAL
2
4
6
8
10
12
14
16
Time (min)

Figure 3.4


A.
Molecular Mass (kDa)
HCT116
HL-60
104
97
50.4
29
DPD
B.
Control
bCD
bCD/UPRT
FBAL (µg/ml)
200
150
100
50
0
24 h Post-Dose

CHAPTER FOUR

Evaluating 5FC Metabolism and the Bystander Effect in Cells Expressing the Fusion Enzyme bCD_{1525}/UPRT

ABSTRACT

The *E. coli* or bacterial enzymes cytosine deaminase (bCD) and uracil phosphoribosyltransferase (bUPRT) can be used in suicide gene therapy for cancer. bCD catalyzes the hydrolytic deamination of the non-toxic prodrug 5-fluorocytosine (5FC) to the chemotherapeutic agent 5-fluorouracil (5FU); bUPRT catalyzes the transfer or a ribosyl-phosphate group to 5FU, producing 5-fluorouridine monophosphate (5FUMP). Endogenous mammalian enzymes then metabolize 5FUMP, generating cytotoxic antimetabolites. However, the poor substrate activity of bCD towards 5FC limits the effectiveness of this therapy. We previously reported that the variant enzyme bCD_{1525} displays increased activity toward 5FC. We sought to further enhance this activity by coupling bCD_{1525} with bUPRT in the fusion construct bCD_{1525}/UPRT. We then analyzed enzyme activity, 5FC metabolism, and cytotoxicity in mammalian cells transfected with bCD_{1525}/UPRT. Additionally, we evaluated the bystander effect to determine if changes in 5FC metabolism lead to a shift in the bystander effect mechanisms. Data suggest a relationship between 5FC metabolism, cytotoxicity, and the BE in C6 rat glioma and HCT116 human colorectal cancer cell lines.

INTRODUCTION

Suicide gene therapy is a promising cancer treatment strategy aimed at limiting systemic toxicity by utilizing prodrug-converting enzymes expressed at tumor sites[1]. One such enzyme is the *E. coli*-derived cytosine deaminase (bCD) which catalyzes the hydrolytic deamination of the prodrug 5-fluorocytosine (5FC) to generate 5-fluorouracil (5FU), a chemotherapeutic agent often used to treat prostate, breast, and colorectal cancers[2]. Endogenous mammalian enzymes further catalyze the conversion of 5FU to 5-fluorouridine monophosphate (5FUMP) and 5-fluorodeoxyrudine monophosphate (5FdUMP), metabolites that interfere with DNA and/or RNA synthesis and processing[3] (Figure 4.1).

A limiting factor of this therapy is the low activity of bCD towards 5FC as compared to its natural substrate cytosine, necessitating the administration of high levels of prodrug to achieve a therapeutic effect. This may result in patient toxicity due to the conversion of 5FC to 5FU by bacteria comprising the normal flora of the gastrointestinal tract[2, 4]. To address this problem, we used regio-specific random mutagenesis to generate bCD variants with higher substrate specificities toward 5FC. We previously reported that the variant bCD_{1525} sensitizes mammalian cells to 5FC, as displayed by a 13-fold reduction in the 5FC IC_{50} value relative to wild-type bCD in HCT116 human colorectal cancer cells[5].

Co-expression of bCD with the *E. coli*-derived enzyme bacterial uracil phosphoribosyltransferase (bUPRT) also increases cell sensitivity to 5FC, likely due to increased 5FUMP concentrations resulting from bUPRT activity toward 5FU[6-12] (Figure 4.1). Several reports indicate that cell sensitivity to 5FC is further increased by

combining bCD and bUPRT into the single fusion construct bCD/UPRT[9, 13]. We hypothesized that this system may be improved by expressing bUPRT as a fusion with bCD_{1525}. We previously demonstrated that concentrations of 5FU are higher in cells expressing bCD_{1525} relative to wild-type bCD[5]. Therefore, we predicted that expression of the bUPRT enzyme would enhance metabolism of the additional 5FU, resulting in higher concentrations of phosphorylated products. Expression of the fusion construct would likely lead to increased fluorinated metabolite incorporation into RNA and DNA as well as increased thymidylate synthase (TS) inhibition by 5FdUMP[8, 13-16].

While elevated concentrations of phosphorylated metabolites may decrease cell survival, they may also affect the bystander effect (BE). The BE, or the transfer of cytotoxic metabolites from transfected cells to untransfected neighbor cells, is vital to achieving complete tumor eradication, especially given the typically low transfection rate of current gene delivery strategies[1, 17]. In the bCD/5FC system, the main BE mechanism is thought to occur via intercellular diffusion of 5FU[18]. For other enzyme/prodrug systems, reports indicate that gap junctions and apoptotic vesicles play critical roles in inducing a BE[19-21]. Gap junctions, or channels connecting the cytoplasmic regions of neighboring cells[22, 23], are permeable to phosphorylated metabolites[24-26], thus allowing movement of cytotoxic metabolites into surrounding cells. Additionally, it has been suggested that phagocytosis of apoptotic vesicles or apoptotic cells by neighboring cells may also contribute to the BE[19]. Although the exact mechanism by which apoptosis contributes to the BE is unclear, possibilities include transfer of cytotoxic metabolites or suicide enzymes within the vesicles or release of pro-apoptotic signals, thus triggering cell death pathways in neighboring cells.

We hypothesize that gap junctions and transfer of apoptotic vesicles may contribute to the BE in cells expressing the fusion enzymes bCD/UPRT and bCD_{1525}/UPRT since it is expected that bUPRT will increase 5FU metabolism, thereby decreasing diffusible 5FU concentrations. Direct comparison of the relative efficacy of the BE mediated by diffusion versus gap junctions or apoptotic vesicles has not been reported. Therefore, it is unclear how a shift in BE mechanisms will effect bystander cell killing. If such a shift is determined to occur upon expression of bCD fusion enzymes, it may offer a good model in which to compare the efficiency of the BE mechanisms. Furthermore, evaluating the BE efficacy will aid in determining the most suitable enzymes for use in suicide gene therapy as it is optimal to use enzymes that both induce cytotoxicity and elicit a robust BE.

MATERIALS AND METHODS

Materials

The radiolabeled substrates for enzyme assays, [^{14}C]-uracil (specific activity, 52 mCi/mmol) and [^{14}C]-5FU (specific activity, 57.2 mCi/mmol), were purchased from Moravek Biochemicals (Brea, CA). Reagents for HPLC and chemicals were purchased from JT Baker (Phillipsburg, NJ). Alamar Blue was purchased from Serotec (Oxford, UK). Other cell culture reagents were purchased from Gibco (Carlsbad, CA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Bacterial strains and genetic complementation

The lysogenized *upp* (UPRT) deficient *Escherichia coli* strain BM604(DE3) [HfrH *thi gale Δ (attλ-bio) deoA103 deoC argA lysA cytR upp udp pyrF30*] was used in genetic complementation studies. To introduce plasmid DNA, 40 μl of electrocompetent *E. coli* cells were transformed with the bacterial expression vector (pETHT) carrying genes encoding the bUPRT, bCD/UPRT, or bCD_{1525}/UPRT enzymes as previously described[27]. Briefly, the transformation mixture was shaken at 37 °C for 1 h in SOC medium (3 g of bactopeptone, 2.5 g of yeast extract, 1 M NaCl, 1 M KCl, 5 mM $MgSO_4$, 5 mM $MgCl_2$, and 1.8% glucose per liter). Cells were concentrated by centrifugation, resuspended in 100 μl SOC and plated onto 2xYT rich medium (for 500 ml- 8 g yeast extract, 5 g trypton, 2.5 g NaCl, 7.5 g agar) supplemented with carbenicillin (50 μg/ml). Following overnight incubation at 37 °C, single colonies were picked and streaked onto positive selection (UPRT) plates. UPRT selection medium (1 L) contained 1.92 g yeast synthetic drop-out without uracil, 100 μl $CaCl_2$,1 mM $MgSO_4$, 11 mM glucose, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4CL, 47 mM Na_2HPO_4, uracil (10 mg/L), thiamin (1 mg/L), biotin (1 mg/L), and carbenicillin (50 μg/ml). Growth on UPRT plates indicates expression of a functional UPRT enzyme. Single colonies were then picked from the UPRT plates, re-streaked onto fresh UPRT plates to confirm the phenotype, and then streaked onto negative selection (5FU) plates to evaluate drug sensitivity. 5FU selection medium was made from UPRT medium supplemented with 5FU (0.5 or 20 μg/ml).

Mammalian cell lines and expression vector

C6 rat glioma cells (ATCC, Manasass, VA) and HCT116 human colorectal cancer cells (a gift from Dr. Neal Davies, Washington State University) were maintained at 37 °C and 5% CO_2 in Dulbeco's Modified Eagle's Medium (DMEM) or McCoy's Medium, respectively, and supplemented with Fetal Bovine Serum at 5 and 10%, respectively. Cells were stably transfected with the mammalian expression vector, pcDNA6/*myc*-HisB (Invitrogen, Carlsbad, CA), carrying genes encoding the enzymes bCD, bCD_{1525}, bCD/UPRT, or bCD_{1525}/UPRT. Cells harboring empty pcDNA vector were used as controls. Transfections were performed using FuGene 6 Transfection Reagent (Roche Molecular Biochemicals) or GeneJuice Transfection Reagent (Novagene®, EMD Biosciences, Inc.) according to manufacturers' recommendations. Positive selection and maintenance of transfectants was accomplished by culturing cells in medium supplemented with blasticidin (4 or 6 μg/ml for C6 and HCT116 cells, respectively).

Enzyme assays

Pools of C6 cells stably transfected with empty vector or pcDNA carrying genes encoding bUPRT, bCD/UPRT, or bCD_{1525}/UPRT were grown to 70% confluency then harvested with trypsin. Cells were lysed via three freeze-thaw cycles in lysis solution consisting of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM dithiothreitol. Total protein was calculated using Bradford Protein Assay (BioRad) with BSA standards. UPRTase activity assays were performed according to lab protocol, as adapted from Lundegaard *et al.*[28] Briefly, lysates (5 μg protein) were pre-incubated at 37 °C for 5

min with 15 μl reaction mix (20 mM TrisCl, pH 8, 5 mM $MgCl_2$, 1 mM GTP, 1 mM phosphoribosyl pyrophosphate). Radiolabeled substrate was added to start the reaction: [^{14}C]-uracil (1 and 10 μM) or [^{14}C]-5FU (5 and 30 μM). The concentrations used correlate with low and mid-range concentrations used for previous UPRT kinetic analysis studies performed in the laboratory. Reactions were incubated at 37 °C for 30 min, stopped by adding 5 μl of 0.33 mM formic acid, and the reaction solution was transferred to DE81 Whatman filter paper, 2.3 cm disks (Whatman, Maidston, England). Discs were washed with deionized water to remove unbound radioactive substrate and air-dried. The amount of radioactive metabolites retained on the discs (i.e., [^{14}C]-UMP or [^{14}C]-5FUMP) was measured using a Liquid Scintillation Analyzer 1900TR (Packard, Prospect, CT).

HPLC analysis of <u>in vitro</u> 5FC metabolite production

Pools of C6 or HCT116 cells stably transfected with bCD enzymes or empty expression vector were seeded on 6-well plates ($1x10^5$ per well) and treated with 5 mM 5FC. Reactions were stopped immediately (Day 0) or on consecutive days, up to 6 days. Reactions were stopped by the addition of 200 μl/well of acetonitrile/acetic acid (94:6) mixture. Cells were collected in media using a cell scraper and counted using a hemocytometer. Cells were lysed by three freeze-thaw cycles at -20 °C and cellular debris removed by centrifugation. 5-Chlorouracil (5ClU) was added to the samples as an internal standard at 100 μg/ml of lysate solution. Samples were then dried to completion using a SpeedVac® Plus SC11A (Savant, Farmingdale, NY), and reconstituted in 1 ml of mobile phase (formic acid:water, 1:99 v/v). Separation of 5FC

and metabolites was carried out on a Phenomenex Luna® $C_{18}(2)$ analytical column (250 mm x 4.6 mm, 5 µm particle size) at a flow rate of 1 ml/min, and metabolites were detected and quantified as previously described[29].

In vitro cytotoxicity assays

Pools of stably transfected C6 or HCT116 cells were seeded in 96 well plates at 500 or 1000 cells/well, respectively, and allowed to attach overnight. Cells were then treated with various concentration of 5FC with the final concentrations ranging from 0-20 mM. Cell viability was determined seven days post-dose using the redox indicator dye Alamar Blue according to the manufacturer's instructions. Cell survival was calculated as percentage of total fluorescence relative to untreated cells. Fluorescence was measured using filters set with excitation/emission wavelength of 530/590 nm on a SynergyHT plate reader using Gen5 analysis software (BioTek Instruments, Inc., Renton, WA).

In vitro analysis of the BE

Pools of stably transfected C6 or HCT116 cells were seeded in 96-well plates at 500 or 1000 cells/well, respectively, and allowed to attach overnight. Cells were then treated with 5 mM 5FC and incubated at 37 °C for four days. The media incubated with the cells ("conditioned media") were collected and transferred to cells expressing empty expression vector (pcDNA). The viability of cells exposed to conditioned media was determined five days post-transfer using Alamar Blue as described above and cell survival calculated relative to untreated cells.

Assessing the role of apoptotic vesicles to the BE

Experiments were set up as described above except prior to transfer of the conditioned media, media was filtered through a MillexGP® 0.22 μm filter unit (Millipore, Billerica, MA). To evaluate the presence of cellular debris in conditioned media, C6 cells carrying empty expression vector were labeled with 5 μM Cell Tracker Green CMFDA Calcein-AM cytosolic dye (Molecular Probes, Eugene, OR). Staining was carried out according to manufacturer's instructions within 1 day of experiments. Labeled cells were seeded in 6-well plates at $2x10^5$ cells per well and allowed to attach overnight. Staining efficiency was estimated by visualizing cells on a Zeiss Observer Z1 fluorescent microscope and AxioVision Release 4.8 software (Zeiss Inc., Göttingen, Germany). Cells were then incubated with 5FU, final concentration of 40 μM in media, for 3 days. Media was then removed and the presence of cellular debris was visualized as fluorescent particulate in the media. Media was passed through a syringe filter with 0.2 μm membrane (Pall Corporation, Ann Arbor, MI) and re-examined for the presence of fluorescent cell matter.

Analysis of gap junction formation

Pools of C6 and HCT116 cells stably transfected with empty expression vector (pcDNA) were labeled with Cell Tracker Green CMFDA Calcein-AM cytosolic cell tracker (Molecular Probes, Eugene, OR) or PKH26 Red Fluorescent Cell Linker membrane dye (Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions no more than 1 day prior to the start of all experiments. Cells were co-cultured at a 1:1

ratio (green:red) in 12-well plates at $2x10^5$ cells/well and allowed to attach overnight. Following a 1 or 4 day incubation at 37 °C and 5% CO_2, cells were visualized using a Zeiss Observer Z1 fluorescent microscope and AxioVision Release 4.8 software (Zeiss Inc., Göttingen, Germany). For flow cytometric analysis of cell populations, cells were collected using trypsin, centrifuged, and resuspended in 100 µl PBS (170 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, and 1.7 mM KH_2PO_4, final pH 7.2). Listmode data was collected on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) and analyzed using FlowJo software version 7.5.5 (Tree Star, Inc., Ashland, OR).

RESULTS AND DISCUSSION

One of the more commonly studied enzyme/prodrug systems for suicide gene therapy is bCD/5FC. The efficacy of this system is hindered by the relatively poor activity of bCD toward 5FC. We previously reported on the isolation of the mutant enzyme bCD_{1525} that displays a shift in substrate preference from cytosine toward 5FC[5]. With the goal of further improving the cell killing ability of bCD_{1525}, we coupled it with bUPRT in the fusion construct bCD_{1525}/UPRT. We hypothesize that this construct will enhance 5FC metabolism thus resulting in increased concentrations of phosphorylated, cytotoxic metabolites. Additionally, we anticipate that an increase in phosphorylated metabolites will result in a shift in the BE mechanism from one primarily involving 5FU diffusion to one more reliant on the transfer of apoptotic vesicles or movement of metabolites through gap junctions.

Evaluation of fusion enzyme activity

Following construction of the fusion enzymes, we tested the relative activity of bUPRT using genetic complementation coupled with plate assays. *E. coli* BM604(DE3) cells were transformed with plasmid DNA harboring the bUPRT, bCD/UPRT, or bCD_{1525}/UPRT genes. Activity of the gene products was evaluated by assessing growth on positive and negative selection plates as described in the Materials and Methods section. As expected, *E. coli* harboring the empty expression vector (pETHT) did not grow on any selection plates but did grow on non-selective (2xYT+carb) plates. As indicated by growth on UPRT plates (positive selection), bUPRT metabolized uracil both as a single enzyme and as part of a fusion construct (Figure 4.2 A). Growth of all cells expressing functional enzymes was restricted slightly on negative selection plates containing 0.5 μg/ml 5FU and was further inhibited on selection plates containing 20 μg/ml 5FU. This suggests that these enzymes are active toward 5FU and that bUPRT expressed as a fusion with bCD or bCD_{1525} is functional (Figure 4.2 B).

<u>In vitro</u> cytotoxicity assays

Pools of C6 and HCT116 cells stably transfected with bCD, bCD_{1525}, bCD/UPRT, bCD_{1525}/UPRT, or empty expression vector (pcDNA) were assessed for activity toward 5FC. As described in the Materials and Methods section, cells were treated with a range of 5FC (0-20 mM) and cell survival was determined following a 7 day incubation period. Table 4.1 shows the 5FC IC_{50} values determined. As previously reported, expression of the wild-type fusion bCD/UPRT decreased the IC_{50} value in C6 cells by

6.8-fold relative to bCD alone[5]. However, bCD/UPRT did not sensitize cells to the extent of bCD_{1525}, as illustrated by the relatively low IC_{50} values for bCD_{1525}. However, HCT116 expressing bCD and bCD/UPRT demonstrated equal IC_{50} values, indicating that addition of the bUPRT enzyme does not further sensitize these cells to 5FC. While it was predicted that expression of bCD_{1525}/UPRT would further sensitize cells to 5FC, we observed only a modest (4-fold) decrease in IC_{50} value relative to bCD_{1525} in C6 cells. Unexpectedly, for HCT116 cells, expression of bCD_{1525}/UPRT increased the IC_{50} value approximately 2-fold compared to expression of bCD_{1525} alone, though this decrease is not statistically significant. These data support previous reports that inherent variations between cell lines and tissues affect the sensitivity to 5FC[2]. We suspect that the variations in the cells' responses to the prodrug may be due to differences in 5FC metabolism. Therefore, we evaluated metabolism of 5FC in cells expressing suicide genes using high-performance liquid chromatography (HPLC).

Analysis of <u>in vitro</u> 5FC metabolism

As described in the Materials and Methods section, we used HPLC to examine production of the 5FC metabolites 5FU and 5FdUMP. C6 and HCT116 cells expressing bCD, bCD_{1525}, bCD/UPRT, or bCD_{1525}/UPRT, or transfected with empty expression vector (pcDNA) were incubated with 5 mM 5FC for 0-6 days. At each time point, cells were collected, counted, and the metabolite concentrations in lysates evaluated using the HPLC method described in Chapter 2. By comparing cell counts, we established growth rates of C6 and HCT116 control cells in the presence of 5FC. When $1x10^5$ C6 and HCT116 control cells were seeded, approximately $7.5x10^4$ and $6.6x10^4$ cells,

respectively, were detected the following day (initial day of 5FC treatment). By day 6 (the final day of 5FC treatment), this number had grown to approximately $1.1x10^6$ and $1.0\ x10^6$ C6 and HCT116 cells, respectively, indicating that these cells grew at relatively the same rate throughout this experiment. Thus, metabolite differences noted between cell lines are likely not due to differences in cell growth.

HPLC analyses revealed that C6 and HCT116 cells expressing bCD_{1525} produced significantly increased concentrations of 5FU relative to control cells on days 3-6 post-dose (Figure 4.3). This correlates with data from previous studies[5] and substantiates the observed increase in cytotoxicity in these cells (Table 4.1). In C6 cells, expression of bCD_{1525}/UPRT resulted in significant 5FU production on days 1-6 compared to control cells (Figure 4.3 A) and compared to bCD_{1525} expressing cells on days 3-6 (Figure 4.3 A, denoted by δ). This counters our prediction that bUPRT expression in fusion constructs would result in decreased 5FU concentrations due to conversion to 5FUMP or 5FdUMP. Therefore, we examined these lysates for the presence of 5FdUMP and detected no differences in 5FdUMP concentrations at any time point between cells expressing single and fusion enzymes.

HCT116 cells expressing bCD_{1525}/UPRT produced significantly higher 5FU levels relative to control cells on days 1-4. On days 5 and 6, the 5FU concentrations in these lysates dropped to levels equal to those detected in lysates of cells expressing bCD/UPRT (Figure 4.3 B). In contrast with C6 cells, 5FU concentrations detected in lysates of HCT116 cells expressing bCD_{1525}/UPRT were not significantly higher than observed in bCD_{1525} expressing cells at any time point. In fact, bCD_{1525} expressing cells produced significantly higher 5FU concentrations on days 5 and 6 relative to

bCD_{1525}/UPRT expressing cells. Again, these data corroborate previous observations that bCD_{1525}/UPRT expression does not sensitize HCT116 cells to 5FC to the extent that bCD_{1525} expression does. However, the lower levels of 5FU may be due to conversion by bUPRT. Therefore, we examined these lysates for presence of 5FdUMP expecting to see increased concentrations in cells expressing fusion enzymes. In HCT116 cells, 5FdUMP concentrations were significantly increased ($p<0.05$) in bCD_{1525} and bCD_{1525}/UPRT expressing cells relative to control cells on days 5 and 6. However, bCD/UPRT expression did not alter 5FdUMP production.

These data may suggest that bUPRT is non-functional in the fusion construct or that perhaps when 5FdUMP binds to the endogenous mammalian enzyme thymidylate synthase (TS), 5FdUMP detection via HPLC is prevented. Plans to evaluate the levels of 5FdUMP-TS complex present in these cell lines using immunoblot assays are described in Chapter 5.

Although we detected bUPRT activity using genetic complementation assays in prokaryotic cells, eukaryotic cells are more complex. Protein expression or stability may differ between prokaryotic and eukaryotic cells and effect enzyme activity. In order to evaluate activity of the fusion enzymes in mammalian cells, lysates of cells transfected with bUPRT, bCD/UPRT, bCD_{1525}/UPRT, or empty mammalian expression vector (pcDNA) were incubated with [C^{14}]-U or [C^{14}]-5FU for 30 min. The presence of radioactive product ([C^{14}]-UMP or [C^{14}]-5FUMP) bound to DE81 filter paper was assessed using a scintillation counter as described in the Materials and Methods section. We expected to detect similar levels of radioactivity in lysates of cells expressing single and fusion enzymes if bUPRT activity was relatively unchanged when

fused to bCD. However, we were unable to distinguish radioactive product from background levels of radioactivity regardless of substrate (uracil or 5FU) or substrate concentration. To try and reduce background, we added cold substrate at 2 and 3-times the concentration of radioactive substrate. We also increased the amount of protein used from 5 μg to 10 or 25 μg (as determined using Bradford assays) but none of these conditions produce detectable radioactive metabolites above background levels, even in cells expressing bUPRT alone. However, others have measured bUPRT enzyme activity and reported active bUPRT when fused with bCD [11, 13, 14, 35]. Therefore, we are evaluating different conditions and protocols in order to evaluate bUPRT activity[10, 11, 13, 14, 35].

Additionally, activity of endogenous enzymes such as uridine kinase and uridine phosphorylase (Figure 4.1) toward 5FU may interfere with detection of bUPRT activity. Therefore, we are also assessing the activity of endogenous mammalian enzymes toward 5FU as compared to bUPRT activity. Future directions of this project are elaborated in Chapter 5.

In vitro analysis of the BE

Since the BE is vital to ensuring complete tumor eradication, it is important that bystander cell killing is not reduced by the expression of variant or fusion enzymes. A conditioned media transfer assay was used to assess the ability of cells expressing bCD, bCD_{1525}, bCD/UPRT, or bCD_{1525}/UPRT to induce bystander cell killing as described in the Materials and Methods section. Enzyme expressing cells were incubated with 5 mM 5FC for 4 days and the resulting conditioned media was

transferred to control cells. Following a 5 day incubation period in conditioned media, control cell survival was assessed and calculated relative to survival of cells incubated in conditioned media lacking 5FC.

We observed little death in control cells exposed to conditioned media from C6 or HCT116 cells transfected with empty vector (pcDNA). However, survival rates of cells transfected with pcDNA decreased when exposed to conditioned media from C6 cells expressing bCD, bCD_{1525}, bCD/UPRT, or bCD_{1525}/UPRT (Figure 4.4 A). This suggests that metabolites produced by these cells elicit a BE, though to varying degrees. Media from C6 cells expressing bCD or bCD/UPRT killed approximately 50% and 20% of control cells, respectively, while media from bCD_{1525} and bCD_{1525}/UPRT expressing cells killed 70% of control cells. There were no significant differences in bystander cell killing between bCD_{1525} and bCD_{1525}/UPRT expressing C6 cells.

In contrast, only HCT116 cells expressing bCD_{1525} or bCD_{1525}/UPRT produced a detectable BE (Figure 4.4 B) and unlike C6 cells, the extent of bystander cell killing is significantly different between these enzymes. In HCT116 cells, bCD_{1525} killed approximately 80% of control cells and bCD_{1525}/UPRT expressing cells killed only 30%. This suggests that as with cytotoxicity and 5FC metabolism, the relative efficacy of the BE may also be cell-line specific, with C6 cells displaying greater 5FC sensitivity and a more robust BE overall than HCT116 cells.

As the BE in the 5FC/bCD system is largely contributed to intercellular diffusion of 5FU, differences in BE efficacy may be explained by examining cell-line specific production of 5FU. C6 cells expressing bCD_{1525} or bCD_{1525}/UPRT produce similar amounts of 5FU and have statistically equivalent levels of bystander cell killing. In

contrast, only HCT116 cells expressing bCD_{1525} produce statistically significant amounts of 5FU and these cell elicited the strongest BE. Therefore, it is likely that the BE observed is dependent mainly on intercellular movement of 5FU.

Assessing the role of apoptotic vesicles in the BE

While the BE most likely results from 5FU diffusion, it has been suggested that transfer of apoptotic vesicles following the death of enzyme-expressing cells may also elicit a BE[19]. As 5FU is known to induce cell apoptosis[31], we examined the contribution of apoptotic vesicles to bystander cell killing using conditioned media transfer assays. As described in the Materials and Methods section, the conditioned media was either transferred directly to control cells or filtered prior to transfer to remove any potentially cytotoxic cellular debris (including apoptotic vesicles). We observed no significant difference in survival levels of control cells exposed to filtered or unfiltered conditioned media (Figure 4.5 A). These data imply that the presence of cellular material does not significantly contribute to the BE. Additionally, it indicates that the BE is mediated by a soluble factor in the media.

To ensure that filtration effectively removed cellular debris, we exposed cells labeled with green fluorescent cytosolic dye to 40 μM 5FU for 3 days as described in the Materials and Methods section. Visualization of these cells on a light microscope suggested that they were undergoing cell death on day 3.

We used fluorescence microscopy to examine surrounding media for fluorescent cellular debris before and after filtration (Figure 4.5 B). On day 0 (first day of 5FU treatment) the cells were labeled with the fluorescent dye (Figure 4.5 B1). Following the

3 day incubation with 5FU, surrounding media contained fluorescent cellular debris (Figure 4.5 B2), but fluorescent particulates were not detected following media filtration (Figure 4.5 B3). In order to confirm these results, we plan to repeat these experiments with an additional step. Following filtration, the cleared conditioned media will be exposed to the red fluorescent membrane dye PKH67 (Sigma-Aldrich, St. Louis, MO). This dye incorporates into lipid regions of cell membranes, allowing staining of any membranes present in the media, including membranes of apoptotic vesicles. Membranes will then be detectable via fluorescence microscopy, thus enabling us to ensure that the filtered media is truly cleared of all cellular debris.

Analysis of gap junction formation

Another mechanism that may contribute to the BE is by passage of phosphorylated metabolites through gap junctions. In order to evaluate gap junctional intercellular communication (GJIC), we examined the intercellular movement of cytosolic dye. C6 and HCT116 cells stably transfected with empty vector (pcDNA) were labeled with either red fluorescent membrane stain (PKH67) or green fluorescent cytosolic stain (Calcein-AM). Following internalization, calcein-AM dye is cleaved by nonspecific esterases, resulting in exposure of carboxylic acid groups, thus preventing passage of dye across cellular membranes via diffusion[36]. However, these compounds are able to move through gap junctions[37]. Therefore, we measured the extent of GJIC by evaluating movement of calein-AM dye into cells labeled with red fluorescent membrane stain and quantified the double-dyed population using flow cytometry as diagramed in Figure 4.6 A[38, 39].

As described in the Materials and Methods section, labeled cells were mixed and seeded in 6-well plates at a 1:1 ratio of red to green cells and incubated in media for four days. Examination of these cells using fluorescence microscopy revealed that following cell seeding and overnight attachment (day 1), the cells were stained, the green and red cells were mixed, and cells were in close enough proximity to form gap junctions. Cells were re-assessed 4 days later. Red and green fluorescent dyes were detectable but a double-dyed cell population was not visible via fluorescence microscopy (Figure 4.6 B). Flow cytometry analysis was used to achieve more sensitive detection and quantification of double-dyed cells. Flow cytometry analyses revealed that approximately 1.4±0.3% of C6 cells were stained with both red and green fluorescent dyes on day 1. This number increased to 9±3.4% by day 4, indicating that intercellular calcein-AM dye movement occurred in a small percentage of C6 cells. However, examination of HCT116 cells revealed that the double-dyed population consisted of only 2.4±0.4% on day 4, signifying less GJIC in these cells. While some reports indicate that even in the presence of low levels of gap junction formation, GJIC can contribute to the BE[40], additional studies are needed to clarify whether the GJIC detected augments the observed BE.

CONCLUSIONS

Overall, we expected that expression of bCD_{1525}/UPRT would decrease cell survival by increasing bUPRT-mediated anabolism of 5FU, thus increasing concentrations of phosphorylated, cytotoxic metabolites and decreasing concentrations of diffusible 5FU. We expected this to result in a shift in the BE mechanisms and

therefore assessed the contribution of apoptotic vesicles to bystander cell killing as well as the extent of GJIC in these cell lines.

In C6 cells, expression of bCD_{1525}/UPRT increased 5FC sensitivity and 5FU production relative to bCD_{1525}, yet the fusion demonstrated relatively equal levels of bystander cell killing. Concentrations of 5FdUMP were not altered in these samples as compared with control cells. In contrast, HCT116 cells expressing bCD_{1525}/UPRT did not display increased 5FC sensitivity or 5FU production and displayed poorer bystander cell killing ability relative to bCD_{1525}. Concentrations of 5FdUMP, however, were increased in both bCD_{1525} and bCD_{1525}/UPRT expressing HCT116 cells. Overall, these data suggest that cell line differences may affect 5FC metabolism and efficacy. Additionally, it suggests a correlation between 5FU concentrations and the BE as cells producing high 5FU levels display a strong BE.

While some reports indicate that apoptotic vesicles may contribute to a BE[19], we demonstrated that the presence of apoptotic vesicles does not contribute significantly to the BE in bCD-expressing cells. Additionally, we assayed the cells for their ability to transfer cytosolic fluorescent dyes through gap junctions and detected low levels of cells participating in GJIC (~10% in C6 and 2% in HCT116 cells). We plan to further assess the contribution of GJIC to the BE in these cells using the chemical β-glycyrrhetinic acid, a general gap junction inhibitor that acts by disrupting gap junction plaque formation[41]. However, given the low concentrations of 5FdUMP produced even in cells expressing fusion enzymes, it is more likely that the majority of the observed BE is due to 5FU diffusion. Even in cells with increased 5FdUMP production, relatively high concentrations of 5FU were detected. This did not support the hypothesis that, in fusion

constructs, bUPRT activity towards 5FU would result in a decrease in diffusible 5FU concentrations. These data imply that wild-type bUPRT expression in mammalian cells does not significantly contribute to 5FC metabolism and is perhaps a good candidate for enzyme engineering studies. Using mutagenesis to improve bUPRT activity toward 5FU may augment the cell killing ability currently demonstrated by bCD_{1525}/UPRT.

Furthermore, these data reinforce the notion that enzymes and enzyme variants may be better suited for treatment of some cancer types over other types. For example, cancers in which express high levels of the endogenous mammalian enzymes uridine phosphorylase and uridine kinase may be sensitive to 5FU without the addition of bUPRT. However, bUPRT may augment therapy better in cancer types with low levels of these endogenous enzymes. A clearer understanding of the relationship between prodrug metabolism, cytotoxicity, and the BE will enable us to direct our research toward designing improved suicide enzymes and more effective prodrug/enzyme combinations for treatment of different cancer types.

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FIGURE LEGENDS

Figure 4.1. 5FC activation pathway in mammalian cells transfected with the *E. coli* genes encoding bCD and bUPRT. Bacterial enzymes are noted in red. bCD, bacterial cytosine deaminase; bUPRT, bacterial uracil phosphoribosyltransferase; udp, uridine phosphorylase; udk, uridine kinase; TS, thymidylate synthase; (5F)C, (5-fluoro) cytosine; (5F)U, (5-fluoro) uracil; (5F)UMP, (5-fluoro) uridine monophosphate; (5F)dUMP, (5-fluoro) deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; dTDP, deoxythymidine diphosphate; dTTP, deoxythymidine triphosphate.

Figure 4.2. Genetic complementation assay of bUPRT activity. The *upp* deficient *E. coli* strain BM604(DE3) was used for genetic complementation assays of bacterial uracil phosphoribosyltransferase activity. Cells were transformed with pETHT, pETHT:bUPRT, pETHT:bCD/UPRT, or pETHT:bCD_{1525}/UPRT and growth assessed on positive (top right panel) and negative (bottom panels) selection plates. Growth or lack thereof indicates bUPRT activity toward uracil and 5FU, respectively. Diagram of plate orientation (1). Growth of enzyme-expressing *E. coli* on positive selection plate (2) or negative selection plate supplemented with 5FU at 0.5 μg/ml (3) or 20 μg/ml (4).

Figure 4.3. Concentrations (μM) of 5FU detected in lysates of C6 (A) and HCT116 (B) cells stably transfected with empty vector (pcDNA), pcDNA:bCD, pcDNA:bCD_{1525}, pcDNA:bCD/UPRT, or pcDNA:bCD_{1525}/UPRT. Data represent metabolite levels (mean±S.E.M.; n=5) following 0-6 day incubation with 5 mM 5FC. Significance was

calculated using a one-way ANOVA test with the Post-Hoc Student-Newman-Keuls Multiple Comparison. Asterisks denote significance relative to pcDNA at *p<0.05, **p<0.005. Significance between bCD_{1525} and bCD_{1525}/UPRT is represented by δ at p<0.001.

Figure 4.4. Analysis of the BE using conditioned media transfer assays. Pools of C6 (A) or HCT116 (B) cells expressing empty vector (pcDNA), pcDNA:bCD, pcDNA:bCD_{1525}, pcDNA:bCD/UPRT, or pcDNA:bCD_{1525}/UPRT were treated with 5 mM 5FC for 4 days. The conditioned media was then transferred to control cells transfected with empty vector. Control cell survival (mean±S.E.M.; n=3) was assessed following a 5 day incubation with conditioned media. Significance was calculated using the student's t-test. Asterisks denote significance relative to pcDNA at *p<0.01, **p<0.0001. Significance between bCD_{1525} and bCD_{1525}/UPRT is represented by δ at p<0.001.

Figure 4.5. Analysis of apoptotic vesicle contribution to the BE. Conditioned media transfer assays (A) were performed as described for the BE analyses (red bars) or conditioned media was filtered (blue bars) prior to transfer to control cells. Results from C6 (left) and HCT116 (right) cells are represented (mean±S.E.M.; n=4). Significance was calculated using the Student's t-test.

Analysis of cellular material removal from conditioned media via filtration (B). Cells were stained with calcein-AM green fluorescent dye and visualized using fluorescence microscopy. Green fluorescent image (B1) of stained cells indicates that the cells were labeled prior to the start of the experiment. Cells were then incubated

with 40 μM 5FU for 3 days to induce apoptosis and the conditioned media was assessed for fluorescent cellular debris (B2). Following filtration through a 0.2 μm membrane, conditioned media was reassessed for fluorescence particulate (B3).

Figure 4.6. Analysis of GJIC. The diagram illustrates the experimental steps for GJIC analysis (A). Cells are labeled with green cytotolic Calcein-AM or red fluorescent membrane PKH67 dye (A1). Green and red labeled cells were mixed (1:1) and were incubated at 37 °C for 4 days to allow movement of cytosolic dye through gap junctions () to occur (A2). Following the 4 day incubation period, cells were collected and assessed for different sub-populations of stained cells (A3). These populations are identified by their relative position on a 2-parameter histogram. Single-red cells are present in the upper left quadrant, single-green in the lower right quadrant, and double-dyed in the upper right quadrant. Cells were visualized using fluorescence microscopy to ensure complete staining and mixing of red and green labeled cells on days 1 and 4 (B).

Table 4.1. 5FC IC_{50} values (mM)±S.D. of C6 and HCT116 cells expressing bCD enzymes as determined by *in vitro* cytotoxicity assays.

5FC IC_{50} Values (mM)		
	C6	**HCT116**
pcDNA	15.5±2.5	16±1.6
bCD	6.8±2.7	11.1±2.0
bCD_{1525}	0.4±0.2	1.6±0.4
bCD/UPRT	1.0±0.7	11.5±3.5
bCD_{1525}/UPRT	0.1±0.05	3.4±1.7

Figure 4.1

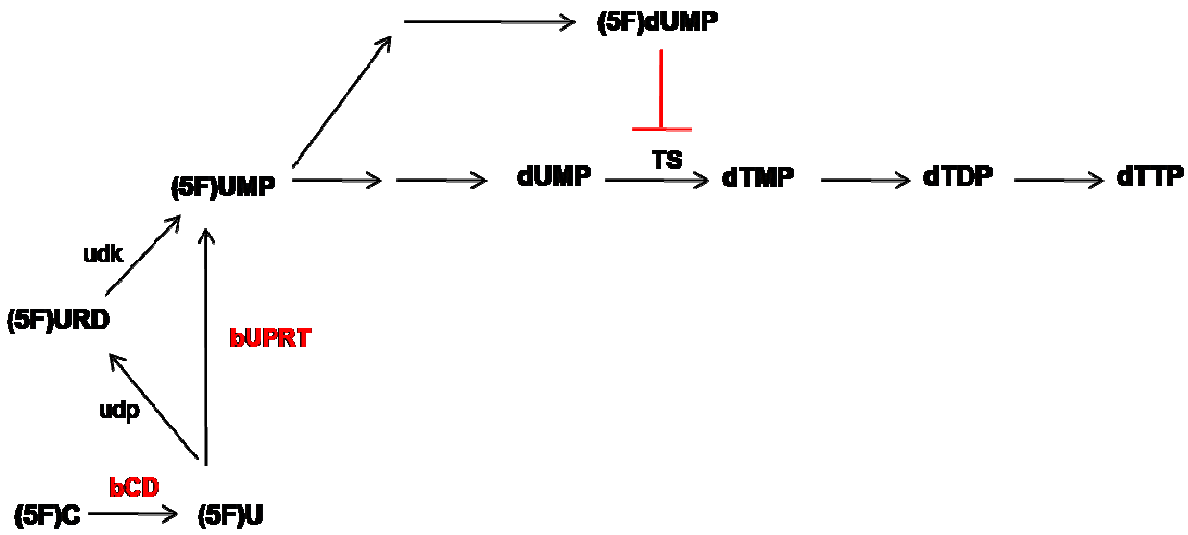

(5F)dUMP
TS
(5F)UMP
dUMP
dTMP
dTDP
dTTP
udk
(5F)URD
bUPRT
udp
bCD
(5F)C
(5F)U

Figure 4.2

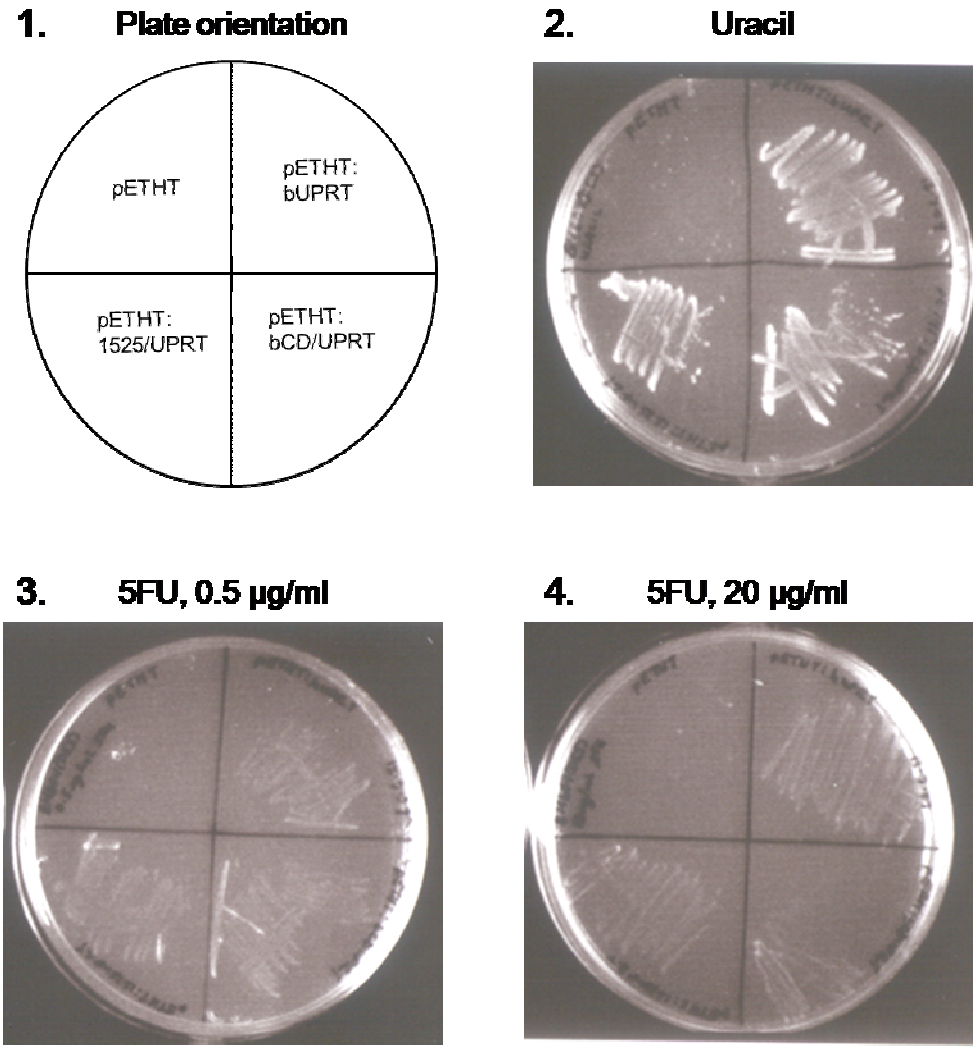

1. Plate orientation
pETHT
pETHT: bUPRT
pETHT: 1525/UPRT
pETHT: bCD/UPRT
2. Uracil
3. 5FU, 0.5 µg/ml
4. 5FU, 20 µg/ml

Figure 4.3

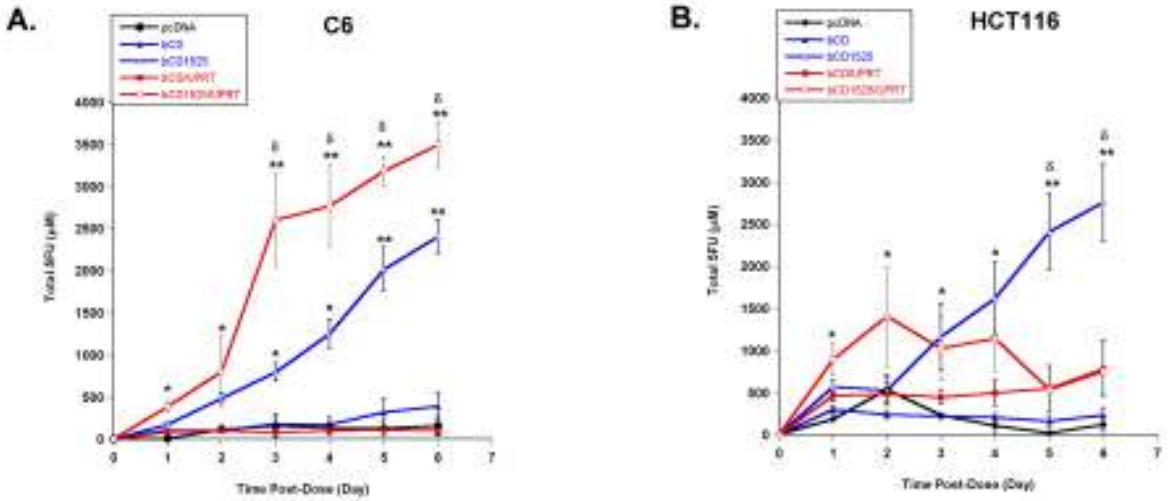

A.
C6
pcDNA
bCD
bCD1526
bCDA/PRT
bCD1526A/PRT
Total SFU (μM)
Time Post-Dose (Day)
B.
HCT116
pcDNA
bCD
bCD1526
bCDA/PRT
bCD1526A/PRT
Total SFU (μM)
Time Post-Dose (Day)

Figure 4.4

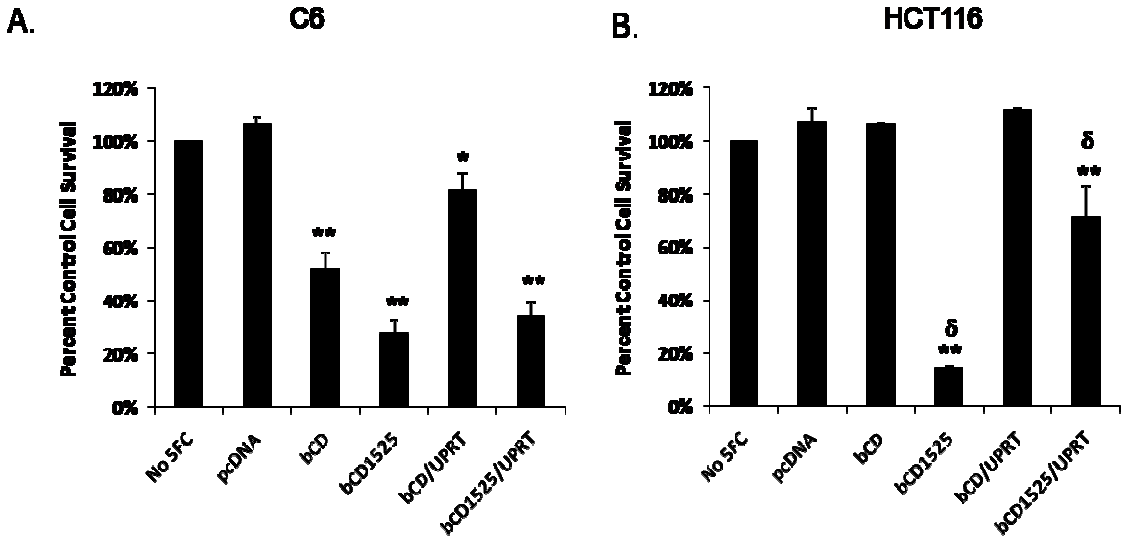

A.
C6
Percent Control Cell Survival
120%
100%
80%
60%
40%
20%
0%
**
**
*
**
No 5FC
pcDNA
bCD
bCD1525
bCD/UPRT
bCD1525/UPRT
B.
HCT116
Percent Control Cell Survival
120%
100%
80%
60%
40%
20%
0%
δ
**
δ
**
No 5FC
pcDNA
bCD
bCD1525
bCD/UPRT
bCD1525/UPRT

Figure 4.5

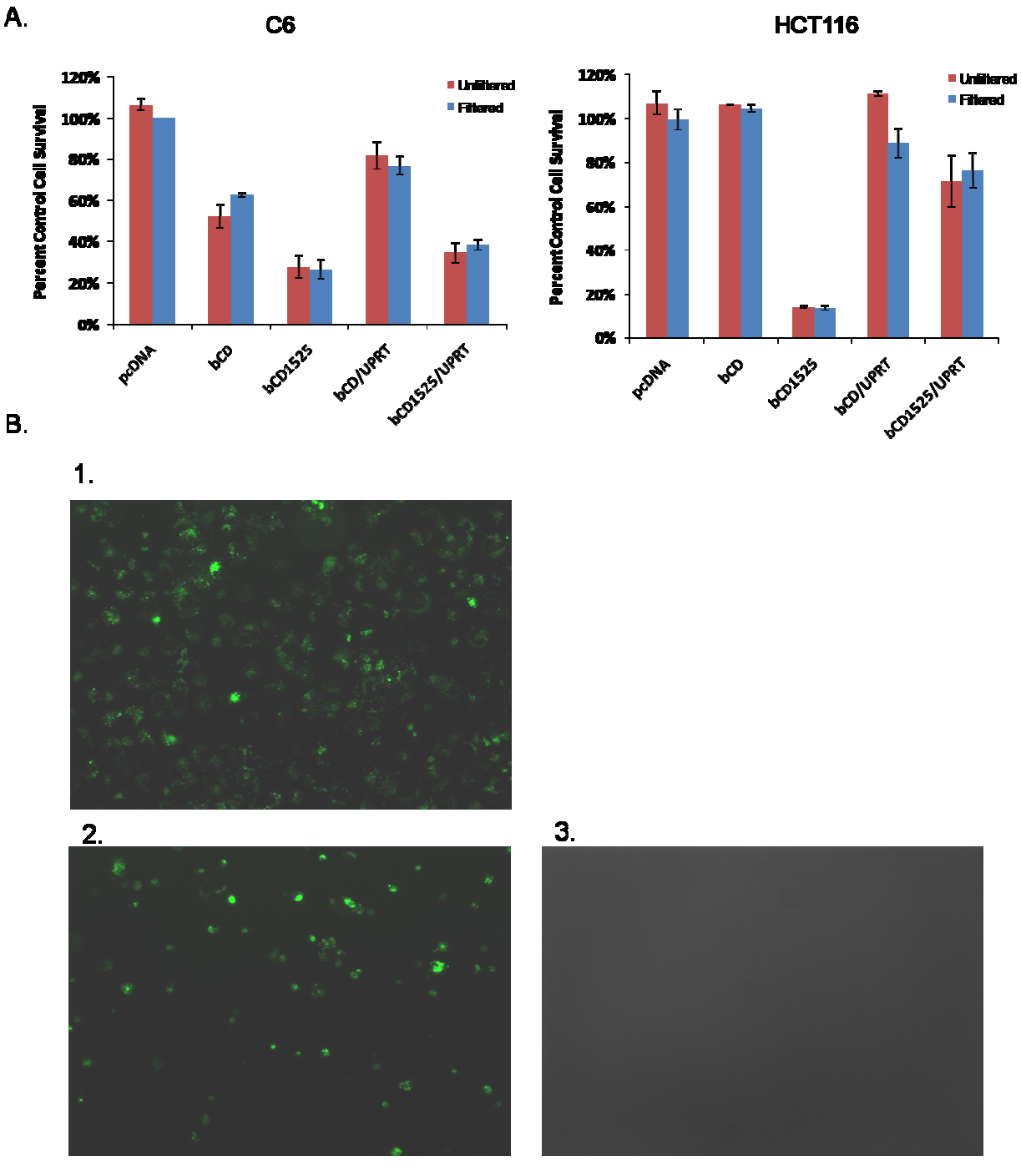
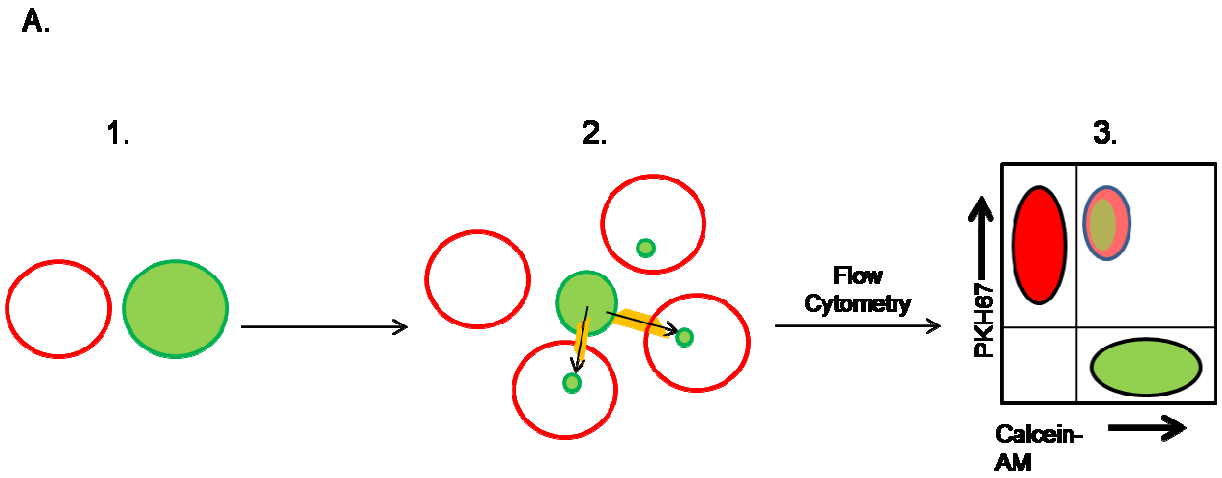
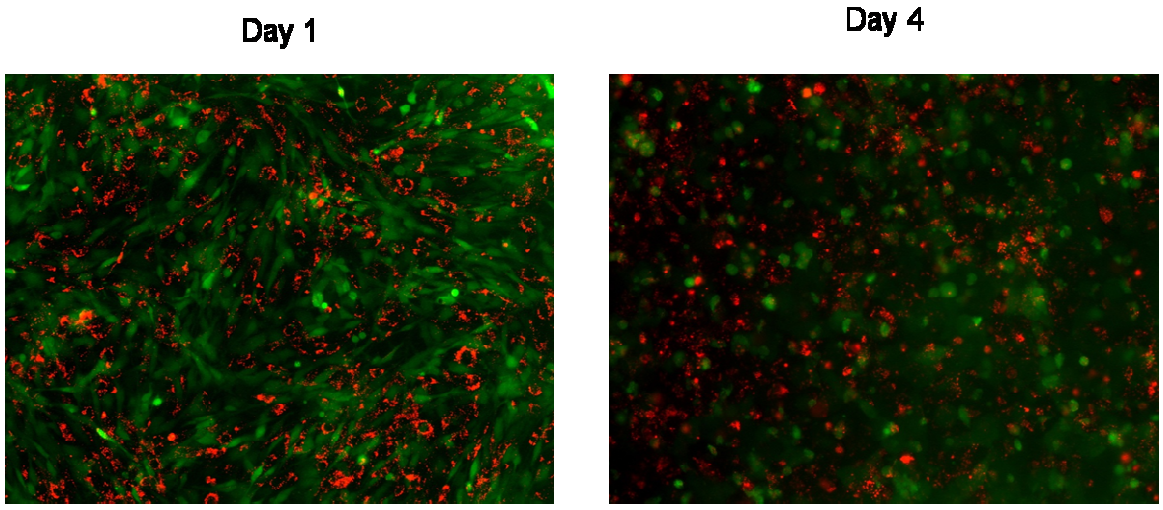

A.
C6
HCT116
Percent Control Cell Survival
120%
100%
80%
60%
40%
20%
0%
Unfiltered
Filtered
pcDNA
bCD
bCD1525
bCD/UPRT
bCD1525/UPRT
B.
1.
2.
3.

Figure 4.6



B.



CHAPTER 5

Summary and Future Directions

Summary

Suicide gene therapy is an alternative cancer treatment strategy aimed at eradicating tumors while limiting systemic patient toxicity by coupling the administration of a non-toxic prodrug with tumor-site expression of prodrug-activating enzymes. Following activation, prodrug metabolites typically cause cell death by interfering with DNA and/or RNA synthesis or causing DNA damage. The overall efficacy of this system is hindered by the lack of a suitable gene delivery system and poor suicide enzyme activity toward prodrug. The research in our laboratory focuses on improving the latter. Increasing enzyme activity will potentially allow less prodrug and/or enzyme to be used to achieve a therapeutic effect, thus limiting prodrug-associated toxicity and immunogenic responses against non-human suicide enzymes.

We have previously used a variety of mutagenesis strategies to generate variant enzymes that enhance cell sensitivity toward prodrug relative to their wild-type counterparts. The molecular basis of this sensitization varies between enzymes but includes improved enzyme activity toward prodrug, decreased enzyme activity toward native substrate, and enzyme thermostabilization[1-5]. Additionally, we have used pathway engineering strategies intended to enhance intracellular prodrug metabolism by co-expressing enzymes responsible for sequential steps in the prodrug activation pathway[6].

Both enzyme and pathway engineering have been used on the *E. coli* derived enzyme bacterial cytosine deaminase (bCD) to improve its activity toward the prodrug 5-fluorocytosine (5FC). In *in vitro* enzyme assays, the mutant bCD_{1525} displayed a 19-fold increase in substrate preference toward 5FC compared to cytosine. This improved activity significantly increased the production of the first metabolite in the 5FC activation pathway, 5-fluorouracil (5FU). Additionally, bCD_{1525} expression in HCT116 cells reduced the 5FC IC_{50} value 17-fold relative to bCD and restricted tumor growth in an *in vivo* model[5]. Others have reported that coupling bCD in an enzyme fusion construct with the 5FU-activating *E. coli*-derived enzyme uracil phosphoribosyltransferase (bUPRT) reduced the 5FC IC_{50} value relative to bCD expression alone in mammalian cells[7-9]. To further enhance this system, we created the fusion construct bCD_{1525}/UPRT and predicted that bUPRT would metabolize the additional 5FU generated by bCD_{1525}, thus increasing cytotoxicity (as discussed in Chapter 4).

When bCD_{1525}/UPRT was expressed in C6 rat glioma cells, we observed a 4-fold decrease in the 5FC IC_{50} value relative to bCD_{1525} alone; however, the IC_{50} value did not change significantly in HCT116 cells expressing bCD_{1525}/UPRT compared to bCD_{1525} alone. To further investigate 5FC metabolism, we developed an HPLC method to detect 5FC metabolites (Chapter 2). This method was used to examine the concentrations of 5FU and 5FdUMP in lysates of C6 and HCT116 cells expressing bCD enzymes. HPLC analyses revealed that in C6 and HCT116 cells expressing bCD_{1525}, 5FU concentrations were significantly increased relative to control cells (Figure 4.3). Expression of bCD_{1525}/UPRT further increased 5FU concentrations in C6 cells but decreased 5FU in HCT116 cells relative to expression of bCD_{1525} alone (Figure 4.3).

Additionally, concentrations of 5FdUMP increased in HCT116 cells expressing bCD_{1525} or bCD_{1525}/UPRT relative to control cells while no detectable changes in 5FdUMP concentrations were noted between any C6 samples. Taken together, these data indicate that variations between cells lines impact suicide enzyme efficacy, perhaps by altering prodrug metabolism, prodrug metabolite metabolism, or cell responsiveness to the final prodrug products.

Another important aspect to consider when creating mutant suicide enzymes is the effect on the bystander effect (BE), or the transfer of toxic metabolites from transfected cells to untransfected neighboring cells. Given the inefficiency of current gene delivery systems, the BE is critical for achieving complete tumor eradication as 80% cell death has been reported following transfection of only 10% of cells[10]. In the bCD/5FC system, it is thought that the BE occurs mainly through intercellular 5FU diffusion[11]. Others have reported that apoptotic vesicle transfer may augment the BE. However, in our studies, the presence of cellular debris, including apoptotic vesicles, did not change the amounts of bystander cell killing (Figure 4.5).

Additionally, movement of metabolites through gap junctions reportedly contributes to the BE, especially in systems that produce metabolites that do not diffuse, such as phosphorylated metabolites. Others have reported that gap junctions contribute to the BE in cells expressing the bCD/UPRT fusion enzyme since bUPRT purportedly increases the concentration of phosphorylated metabolites[12]. An evaluation of dye transfer in the C6 and HCT116 cell lines revealed that <10% of these cells participate in GJIC (Chapter 4) but it remains unclear if, in these cells, the BE is augmented by gap junctions. Overall, our data suggested a correlation between 5FC

metabolism, cytotoxicity, and the BE as cells that produced the highest concentrations of 5FU also displayed the most sensitivity to 5FC and the highest level of bystander cell killing. Discussed below are the plans to further examine this relationship.

Future Prospects

Enzyme Activity and 5FC Metabolism

Following construction of the fusion enzyme bCD_{1525}/UPRT, we used genetic complementation and plate assays to assess bUPRT activity toward uracil and 5FU (Figure 4.2). These experiments indicated that bUPRT is active when expressed alone or in fusion with bCD or bCD_{1525}. To further evaluate bUPRT activity in mammalian cells, we performed enzyme assays using lysates of C6 cells stably transfected with bUPRT, bCD/UPRT, bCD_{1525}/UPRT, or empty expression vector (pcDNA), as described in Chapter 4. We were not able to distinguish bUPRT activity due to high levels of background in these assays. We are continuing to evaluate different experimental conditions in order to rectify this problem.

HPLC analyses of 5FC metabolism revealed no significant 5FdUMP production upon expression of fusion constructs in C6 cells. In HCT116 cells, 5FdUMP concentrations were slightly increased compared to concentrations in controls following a 5 or 6 day incubation period with 5FC. As suggested in Chapter 4, the HPLC method used may not be able to detect changes in 5FdUMP concentrations if 5FdUMP was bound in complex with thymidylate synthase (TS). Therefore, we plan to evaluate the levels of bound and unbound 5FdUMP in cells exposed to 5FC using immunoblot assays and anti-TS antibody (Rockland, Gilbertsville, PA). Upon formation of the TS-

5FdUMP complex, a shift in protein band size from 36 kDa (unbound TS) to 38 kDa is detectable[13]. Protein levels will be quantified relative to a loading control in order to determine the relative amounts of TS-5FdUMP complex formed in cells expressing wild-type or variant single and fusion enzymes. Additionally, we will use immunoblot analysis to evaluate the endogenous levels of TS in C6 and HCT116 cells. Cell line variations of TS protein may account for the observed differences in 5FC sensitivity. Cells that express increased levels of TS are relatively resistant to 5FC as 5FdUMP does not bind all the available TS, thereby allowing cells to recover from 5FC treatment[14-17]. The role of TS in 5FdUMP binding in these samples can be further evaluated using TS binding assays, as previously described[18, 19].

The lack of detectable changes in 5FdUMP concentrations in HPLC analyses and enzyme assays may also stem from high endogenous enzyme activity toward 5FU, resulting in high background. Uridine phosphorylase (udp) and uridine kinase (udk) catalyze 5FU conversion to 5-fluorouridine (5FURD) and 5-fluorouridine monophosphate (5FUMP), respectively (Figure 1.2), but it is unclear how this activity compares with bUPRT activity. Furthermore, it has recently been suggested that mammalian cells may express a UPRT enzyme[20], though its expression within different cell lines has not been determined. To evaluate the activity of endogenous mammalian enzymes toward 5FU, we will use HPLC to evaluate 5FU metabolism in untransfected C6 and HCT116 cells. These experiments will be designed as previously described for evaluating 5FC metabolism (Chapter 4) except we will incubate cells with 5FU in place of 5FC. These experiments will aid us in elucidating the extent that endogenous mammalian enzymes activate 5FU.

Additionally, we plan to examine 5FU metabolism in C6 and HCT116 cells transfected with bUPRT, bCD/UPRT, bCD_{1525}/UPRT, or empty vector. These experiments will allow us to directly compare the relative activity of bUPRT toward 5FU when expressed in mammalian cells as a single or fusion construct and may clarify whether bUPRT is active as a fusion construct.

Another mammalian enzyme that may interfere with 5FdUMP formation is dihydropyrimidine dehydrogenase (DPD). DPD catalyzes the conversion of 5FU to 2-fluoro-β-alanine (FBAL), potentially limiting the amounts of 5FU substrate available for bUPRT (as discussed in Chapter 3). HPLC analysis of FBAL formation in HCT116 cells suggested that these cells express active DPD enzyme; however, activity was not established in C6 cells. Using the HPLC method described in Chapter 3, we plan to assess DPD activity in C6 cells as well as FBAL production upon expression of bUPRT alone or fused with bCD or bCD_{1525}. We expect to gain a more complete understanding of *in vitro* 5FC metabolism by using HPLC to examine both FBAL formation and phosphorylated metabolite production upon expression of wild-type or variant single and fusion enzymes.

Data from these experiments will aid in determining which suicide enzymes may be the most effective for use in suicide gene therapy. For example, if expression of bUPRT decreases DPD-mediated catabolism of 5FU, it may be more effective to use fusion enzymes than single enzymes to treat tumors in which DPD is up-regulated. In contrast, if bUPRT expression is ineffective at metabolizing 5FU in the presence of DPD or if its activity toward 5FU is not significantly higher than uridine phosphorylase (udp) and uridine kinase (udk) activity, it may indicate a need for an improved UPRT enzyme.

An ongoing project in our laboratory is to perform mutagenesis on bUPRT with the goal of generating enzymes that are more active toward 5FU[21], thereby, potentially increasing 5FU metabolism to cytotoxic metabolites.

The Bystander Effect

As part of our future work, we plan to further investigate the mechanisms of the BE. We hypothesized that expression of fusion enzymes would increase the production of phosphorylated metabolites while decreasing concentrations of 5FU. Thus, we anticipated that the mechanism of the BE would shift from 5FU diffusion to intercellular movement of phosphorylated metabolites through gap junctions or transfer of apoptotic vesicles. However, as detailed in Chapter 4, HPLC analyses revealed that C6 cells expressing bCD_{1525}/UPRT produced the highest 5FU concentrations (Figure 4.3 A), but we did not detect significant changes in the 5FdUMP concentrations between C6 cells expressing fusion or single bCD enzymes. On the other hand, HCT116 cells expressing bCD_{1525}, not bCD_{1525}/UPRT, produced the highest 5FU concentrations on days 3-6 (Figure 4.3 B), though both of these enzymes produced increased concentrations of 5FdUMP on day 6 relative to control cells. This contradicted our hypothesis that cells expressing fusion enzymes would produce higher 5FdUMP levels and lower 5FU levels compared to single enzymes.

When we evaluated the BE using conditioned media transfer assays, we determined that all C6 cells expressing bCD suicide enzymes elicited a BE, though to varying degrees. Levels of bystander cell killing were the highest in cells expressing bCD_{1525} or bCD_{1525}/UPRT and were not significantly different between these enzymes.

On the other hand, only HCT116 cells expressing bCD_{1525} or bCD_{1525}/UPRT elicited a BE and it was significantly different between these enzymes. For both C6 and HCT116 cells, the enzymes that produced the highest 5FU concentrations induced the highest amounts of bystander cell killing, suggesting that the BE in these cells is mediated by 5FU diffusion. Additionally, filtering the conditioned media to remove cellular debris did not significantly alter the levels of bystander cell killing (Figure 4.5), further supporting the idea that the BE is induced by a soluble factor(s) present in the media.

While it is likely that the BE is 5FU-mediated, it was important to assess the extent of GJIC in these cells since our future investigations are aimed at improving bUPRT activity toward 5FU, potentially resulting in increased 5FdUMP and decreased 5FU concentrations. By using fluorescent dyes coupled with flow cytometry, we detected GJIC in a small population of HCT116 and C6 cells, 3% and 10%, respectively. It is unclear whether these low levels of GJIC will contribute to a BE. Nevertheless, should concentrations of phosphorylated metabolites be increased by a mutant bUPRT enzyme, it will be important to assess the gap junction contribution to the BE. This will be accomplished by treating cells with β-glycyrrhetinic acid, a chemical inhibitor of gap junctions[22], prior to performing conditioned media transfer assays. If treatment with β-glycyrrhetinic acid does not alter levels of bystander cell killing, it will suggest that gap junctions do not contribute to the BE in these cell lines. Additionally, we may be able modulate the level of GJIC by co-transfecting these cells with connexin-encoding genes along with the suicide enzymes. Others have demonstrated an increase in BE following up-regulation of connexins[23-26]. Since the BE is vital to

achieving complete tumor eradication via suicide gene therapy, we must ensure that our mutant enzymes will elicit a robust BE.

In conclusion, we developed and validated two novel HPLC methods that were used for the separation, detection, and quantification of various 5FC metabolites in cell culture samples. These methods were used to assess 5FC metabolism in C6 and HCT116 cells transfected with the *E. coli* genes encoding bCD, bCD_{1525}, bCD/UPRT, or bCD_{1525}/UPRT. These methods will be employed in future studies to evaluate 5FC metabolism by endogenous mammalian enzymes as well as to establish DPD activity in C6 cells and to assess the role of DPD and bUPRT enzymes in metabolizing 5FU. These studies will enable us to better understand the *in vitro* activity of wild-type and variant enzymes and determine the relative activity of mammalian enzymes toward prodrug and prodrug metabolites.

Furthermore, we determined that 5FU diffusion is likely the major mode of the BE in C6 and HCT116 cells, even when fusion enzymes are expressed. However, we anticipate that the main BE mechanism may change in the presence of a mutant bUPRT enzyme with improved activity toward 5FU. In general, this research will enhance our understanding of the relationship between prodrug metabolism and the BE as well as the effect of mammalian enzymes on prodrug efficacy. This knowledge will enable us to direct our research toward improving enzymes that inadequately metabolize prodrug as well as engineering enzymes that better elicit a BE or limit prodrug metabolism by endogenous mammalian enzymes. By improving suicide enzymes, we aim to make suicide gene therapy a more effective and realistic cancer treatment strategy.

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