SUICIDE GENE THERAPY FOR CANCER

by

MADHURA SPANDANA TUMMALAPALLY

(Under the Direction of Dexi Liu)

ABSTRACT

Suicide gene therapy is a treatment strategy designed to enhance the efficacy of drug therapy of cancer by targeting cytotoxic drugs to the tumor but not normal cells. It involves delivery of enzyme coding gene to tumor cells where the expressed enzyme can convert a nontoxic prodrug molecule once entering the cell to toxic drugs leading to apoptosis and cell suicide. The enzyme-prodrug pairs being explored include HSV-TK/GCV, CD/5-FC, NTR/CB1954, CYP450/Cyclophosphamide, and CPG2/CMDA. Enzyme coding genes are cloned into plasmid or packaged into vectors that facilitate gene transfer into target cells. Following prodrug administration, cytotoxic effects are seen in all tumor cells through a mechanism of bystander effect. The focus of this thesis is to provide a introduction on cancer biology, and the treatment strategies currently available for cancer therapy. A major emphasis will be placed on the approaches of suicide gene therapy against cancer with detailed rationale of each approach, current progress and future perspectives for their clinical applications.

INDEX WORDS: Prodrug, Cancer, Suicide gene therapy, Enzymes, Vectors, Bystander effect

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DEDICATION

Every challenging work needs self-efforts as well as the guidance of elders, especially closed ones.

This thesis is dedicated first and foremost to me. I never expected, in a million years that I would arrive at this juncture of my life. I also dedicate this to my family, especially my mother and sister, Mani Nutulapati and Haritha Chandana. Their affection, love, encouragement, and support made me achieve this success and honor.

I also dedicate this to all the hardworking and respectable professors who have guided throughout my journey and polished me well.

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Finally, I must express my very profound gratitude to my family, for providing me with unfailing support and boosting up my confidence when I needed the most.

LIST OF ABBREVIATIONS

ABBREVIATION	TERM
5-FC	5-Fluorocytosine
5-FdUMP	5- Fluorodeoxyuridine Monophosphate
5FdUTP	5-Fluorodeoxyuridine Triphosphate
5-FU	5-Fluorouridine
5-FUTP	5-Fluoro-Uridine Triphosphate
AAV	Adeno-Associated Virus
ANM	Aniline Nitrogen Mustards
BANM	Benzoic Acid Nitrogen Mustards
BBB	Blood Brain Barrier
BCC	Breast Cancer Cells
BTB	Blood Tumor Barrier
CAR	Coxsackie and Adenovirus Receptor
CB1954	(5-(Aziridin-1-Yl)-2,4-Dinitrobenzamide)
CD	Cytosine Deaminase
CMDA	4-([2-chloroethyl][2-mesyloxyethyl]amino)benzoyl-L-glutamic acid
CNS	Central Nervous System
CPA	Cyclophosphamide
CPG2	Carboxypeptidase G2
CPNP	Cosmetic Products Notification Portal
CYP450	Cytochrome P450
dGTP	Deoxyguanosine Triphosphate
DOPE	Dioleoylphosphatidyl Ethanolamine
DOTAP	1, 2-Dioleoyl-3-Trimethylammonium-Propane

DsRed	*Discosoma Sp.* Red Fluorescent Protein
dsRNA	Double-Stranded RNA
dTTP	Deoxythymidine Triphosphate
EPC	Endothelial Progenitor Cells
EPR	Enhanced Permeability and Retention
FACS	Fluorescence-Activated Cell Sorting
FADD	Fas-Associated Death Domain
GCV	Ganciclovir
GDEPT	Gene Directed Enzyme Prodrug Therapy
HIV	Human Immunodeficiency Virus
HMEC	Human Mammary Epithelial Cells
HSV	Herpes Simplex Virus
IFA	Ifosfamide
LOI	Loss of Imprinting
LTR	Long Terminal Repeats
MDR1	Multidrug Resistance-1
mDsRed	Monomeric Dsred
MLV	Murine Leukemia Virus
MPEG-PCL	Methoxypoly(Ethylene Glycol) Poly(Caprolactone)
mRNA	Messenger RNA
MSC	Mesenchymal Stem Cells
NSC	Neural Stem Cell
NTR	Nitroreductase
OS	Overall Survival
PBMC	Peripheral Blood Mononuclear Cells
PDMAEMA	Poly[2-(N,N-Dimethylamino)Ethyl Methacrylate]
PEI	Poly(Ethyleneimine)
PFS	Progression Free Survival
PLL	Poly-L-(Lysine)

PNM	Phenol Nitrogen Mustards
RNAi	RNA Interference
RV	Retroviral
SCID	Severe Combined Immunodeficient
shRNA	Short Hairpin RNA
siRNA	Small Interfering RNA
TK	Thymidine Kinase
TTP	Time to Progression
UPRT	Uracil Phosphoribosyltransferase
VEGF-A	Vascular Endothelial Growth Factor A

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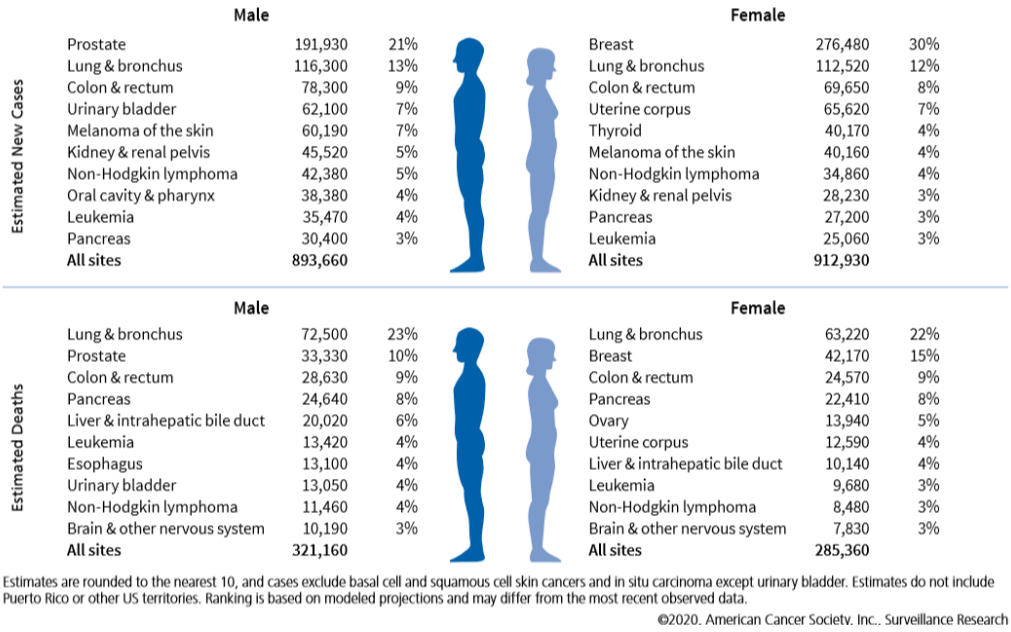
CHAPTER 1 - BASICS OF CANCER

Cancer can be described as a disease caused by uncontrolled growth of cells in the body. It is one of the major leading causes of morbidity and mortality worldwide, with 1 in every 6 deaths. In 2018, there were an estimated 17 million cases of cancer diagnosed around the world and 9.5 million cancer deaths. By 2040, the global burden is expected to reach 27.5 million cancer cases and 16.2 million cancer deaths due to population growth and aging [1]. In the US, an estimated 40 out of 100 men and 39 out of 100 women will develop cancer during their lifetime. Figure 1 shows the estimated new cases and death rates of major types of cancer in the US. More than 1.8 million new cancer cases are expected to be diagnosed in 2020.



Estimated New Cases

Male			Female		
Prostate	191,930	21%	Breast	276,480	30%
Lung & bronchus	116,300	13%	Lung & bronchus	112,520	12%
Colon & rectum	78,300	9%	Colon & rectum	69,650	8%
Urinary bladder	62,100	7%	Uterine corpus	65,620	7%
Melanoma of the skin	60,190	7%	Thyroid	40,170	4%
Kidney & renal pelvis	45,520	5%	Melanoma of the skin	40,160	4%
Non-Hodgkin lymphoma	42,380	5%	Non-Hodgkin lymphoma	34,860	4%
Oral cavity & pharynx	38,380	4%	Kidney & renal pelvis	28,230	3%
Leukemia	35,470	4%	Pancreas	27,200	3%
Pancreas	30,400	3%	Leukemia	25,060	3%
All sites	**893,660**		**All sites**	**912,930**	

Estimated Deaths

Male			Female		
Lung & bronchus	72,500	23%	Lung & bronchus	63,220	22%
Prostate	33,330	10%	Breast	42,170	15%
Colon & rectum	28,630	9%	Colon & rectum	24,570	9%
Pancreas	24,640	8%	Pancreas	22,410	8%
Liver & intrahepatic bile duct	20,020	6%	Ovary	13,940	5%
Leukemia	13,420	4%	Uterine corpus	12,590	4%
Esophagus	13,100	4%	Liver & intrahepatic bile duct	10,140	4%
Urinary bladder	13,050	4%	Leukemia	9,680	3%
Non-Hodgkin lymphoma	11,460	4%	Non-Hodgkin lymphoma	8,480	3%
Brain & other nervous system	10,190	3%	Brain & other nervous system	7,830	3%
All sites	**321,160**		**All sites**	**285,360**	

Estimates are rounded to the nearest 10, and cases exclude basal cell and squamous cell skin cancers and in situ carcinoma except urinary bladder. Estimates do not include Puerto Rico or other US territories. Ranking is based on modeled projections and may differ from the most recent observed data.

Figure 1 2020 Cancer Estimates

Although the causes of cancer are not fully understood, many factors have been identified for their role in increasing the disease's occurrence [Figure 2], including environmental and genetic factors. It is known that environmental factors such as diet, tobacco, viral infection, alcohol among others influence cancer development and progression. These factors induce epigenetic changes involving histone modifications, DNA methylation, and loss of imprinting (LOI) leading to expression of genes responsible for growth. Approximately 90-95% of known cancers are caused by environmental factors. Physiological status such as obesity and inflammation has also been linked to cancer. Genetic factors responsible for an estimated 5-10% of total cancers are linked to accumulating mutations in DNA sequence resulting in an elevated expression of oncogenes or decreased expression of tumor suppresser genes. It is more common that the epigenetic and genetic factors could act simultaneously or in sequence to initiate and/or promote cancer development.

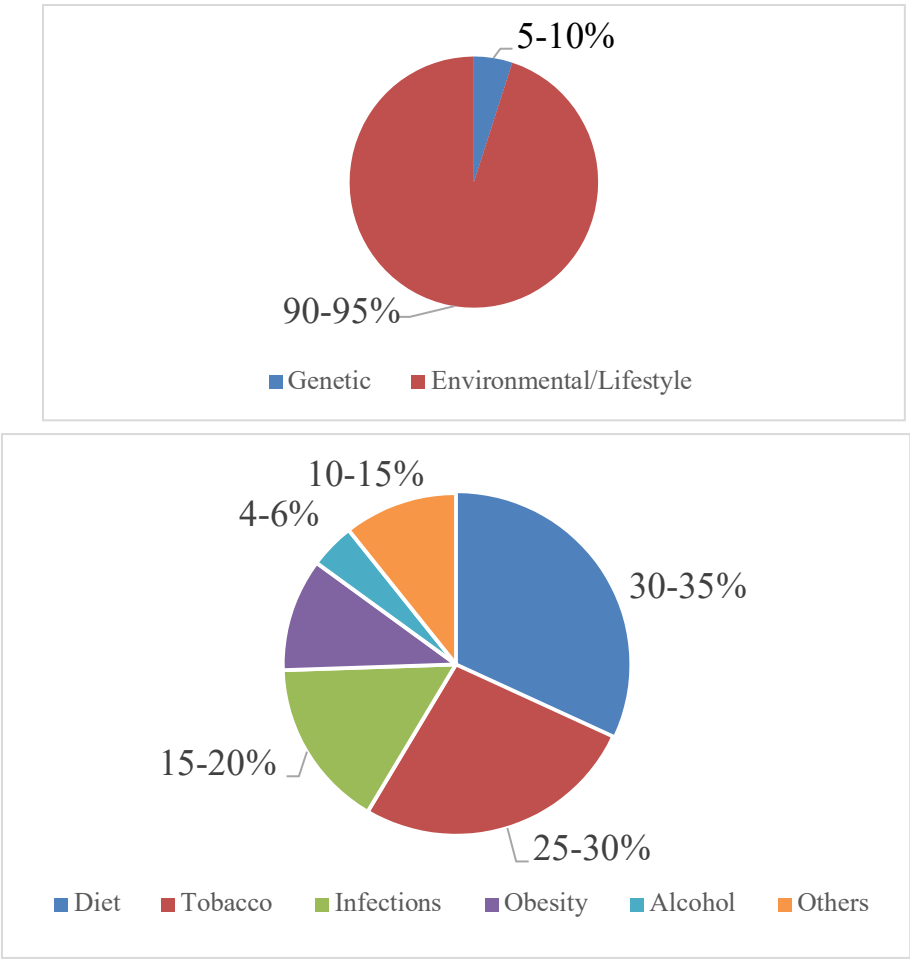


Figure 2 Causative Factors of Cancer

The guiding principle for cancer treatment in the past has been to stop tumor growth or eliminate cancer cells from the body. Surgery, radiation therapy and chemotherapy constitute the conventional treatment. Surgery is the most effective in the treatment of localized primary tumor and associated regional lymphatics. When used, single treatment of surgery cures more patients than any other forms of therapy because surgery could excise 100% tumor cells if identified and accessible. Major limitation of surgery-based approach is that not all tumor cells are surgically removable without risking tissue damage that could be detrimental for normal function. In

addition, surgery is not practical in treating cancers with wide spread of metastasis. Radiotherapy, developed in 1920s, uses radiation energy to induce DNA damage and apoptosis of tumor cells. It is commonly used as a curative therapy for early stage head and neck tumor, prostate cancer, and Hodgkin's disease. In addition, preoperative radiation therapy is also used in rectal and esophageal carcinomas, and postoperative radiation therapy is used in breast and central nervous system tumors. Radiation therapy is an important means to provide a rapid and effective palliation to local and/or metastatic diseases such as bone or cerebral metastasis. The major limitation of radiation therapy, however, is that it can kill a fraction of tumor cells by each treatment without sparing nearby normal cells. Chemotherapy began in the 1940s. Chemotherapeutic drugs are designed to disrupt functions of tumor cells such as DNA replication, cell cycling, or critical signal transduction pathways. Comparing to surgical and radiation therapy, the last century has seen a significant progress in chemotherapy. A major advantage of chemotherapy is that anticancer drugs, once systemically administered, can reach tumor cells at the primary and metastatic tumor sites. The drawback of systematic drug therapy is that normal cells in essential organs are also exposed, leading to side effects that are sometime life threatening. In recent years, antibody-based therapy or immunotherapy was introduced for management of cancer patients and has gained FDA approval for the treatment of lymphoma using rituximab antibodies [2]. Recent success of immunotherapy has made this approach as the first choice in many cases. While successful under different conditions for each of the specific strategies, the therapeutic modalities developed so far are often applied in combination with each other to enhance the efficacy. Despite the new developments and advances in cancer therapy made in the past century, mortality caused by most of the solid tumors has not changed substantially. Partly responsible for these failures are the lack of tumor specificity. Therefore, as most cancers remain to give a less than full response to the

current therapeutic options, the development of target specific and more efficient strategies is needed.

Targeted therapy represents a new strategy that aims at cancer cells but spare toxicity to off- target normal cells. The success of the treatment lies in the targeted release of therapeutics at the disease site to block a specific pathway or enzyme unique to cancer cells including cell proliferation, cell cycle checkpoints, pathways of apoptosis or autophagy [3]. This therapy often involves the use of monoclonal antibodies or ligands that recognize surface properties of tumor cells [4]. On the other hand, gene therapy appears as a good alternative and holds a great promise for the treatment of cancer and is evidenced by a huge number of clinical trials [5–10]. Different gene therapy strategies have been explored including mutation correction, enhancement of the immune response against tumor cells, RNA interference, targeted lysis of tumor cells using selective replicative viruses, and anti-angiogenic and suicide gene therapies [11]. In this thesis, I will focus on suicide gene therapy focusing on principles of different approaches and the current progress toward their clinical applications.

CHAPTER 2 - CONCEPT OF SUICIDE GENE THERAPY AND SYSTEMS DEVELOPED

Suicide gene therapy, also known as gene directed enzyme prodrug therapy (GDEPT), is a strategy for cancer treatment based on an introduction into and expression of a viral or bacterial gene in tumor cells, which allows the conversion of a non-toxic compound into a lethal drug [Figure 3]. In the first step, the enzyme-coding gene is cloned into a vector and delivered to tumor cells with a carrier. In the second step, the gene is transcribed and translated in the tumor cell. In the third step, a prodrug is administered systemically and taken up by the cells. The prodrug molecules will only be converted to cytotoxic drug by the enzymes in the cancer cells to which the therapeutic gene has been delivered. Tumor specific expression of prodrug conversion enzymes is normally achieved using a target ligand recognizing membrane proteins on tumor cell surface or by using tumor specific promoter driving the expression of enzyme coding gene. Normal cells,

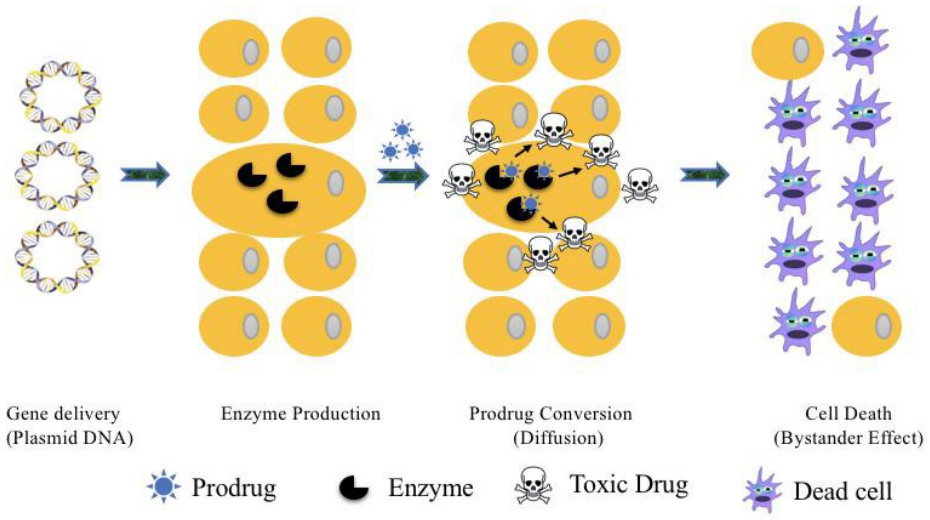


Figure 3 Mechanism of Suicide Gene Therapy (Modified from Malekshah et al., 2016)

due to lack of specific surface structure or/and tumor specific transcription factor, will not be able to produce prodrug conversion enzymes and therefore are spared for cytotoxicity.

Significant efforts have been made in the past two decades searching for the most appropriate suicide genes, establishment of effective delivery vehicles, and development of prodrugs highly effective in killing cancer cells. Many of the studies were conducted at cellular level, some are in animals and a few in humans. The following are the most commonly studied enzyme/prodrug systems.

2.1. Herpes Simplex Virus Type 1-Thymidine Kinase/Ganciclovir (HSV-TK/GCV) System

HSV-TK/GCV system is the first system studied for suicide gene therapy and has been extensively studied. GCV is used as a prodrug, and thymidine kinase can phosphorylate GCV to GCV triphosphate, an analog of guanine-5-triphosphate and the natural substrate of DNA polymerase. Lack of ribose structure in GCV prevents DNA chain elongation [12-14].

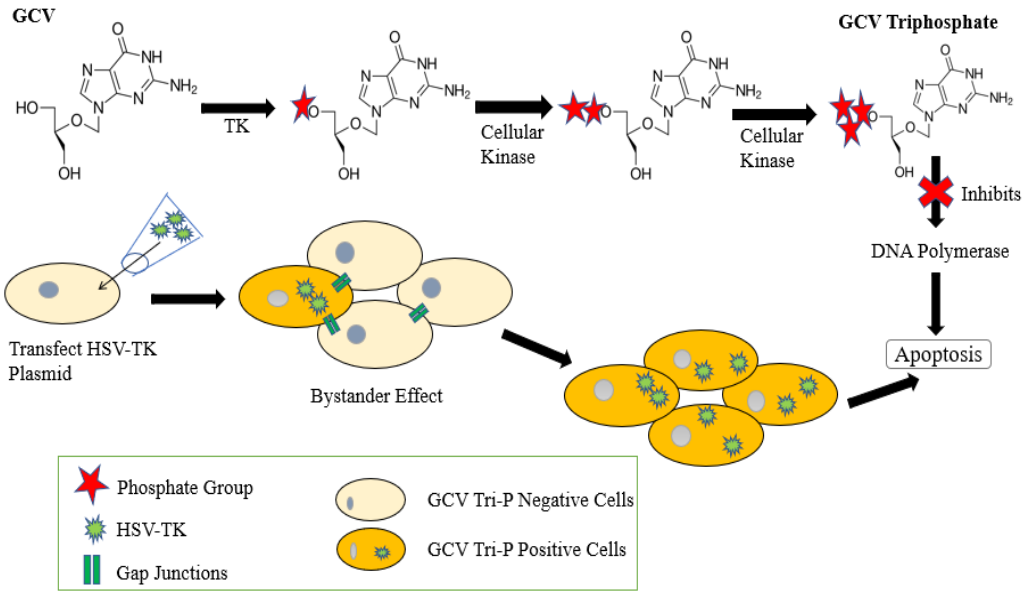


Figure 4 Mechanism of Ganciclovir in Tumor cells (Modified from D Dey., 2011)

S-phase delay and G-2 phase arrest were observed due to the activation of exonuclease and post-replicative endonuclease repair mechanisms when GCV triphosphate was erroneously incorporated into the DNA [15–18]. Wei et al. concluded that apoptosis was involved in this GCV-induced cell cycle arrest [19].

In a study, the relevance of the cell cycle control towards the sensitivity of pancreatic tumor cells to the cytotoxicity induced by the HSV-TK/GCV system was demonstrated, where it was observed that the Chk1 activation was associated with a greater HSV-TK/GCV extent of cell death [23]. In-vivo anti-tumor activity of the TK/GCV system has been showed in several carcinoma animal models, including leukemia [24], glioma [25,26], bladder cancer [27], intrahepatic metastasis of liver cancer [28], colon adenocarcinoma [29], and oral cancer, [30,31].

Pre-clinical studies showed some positive results with the HSV-TK/GCV system leading to its application in a number of clinical trials for many types of cancer [32–37]. In a phase I/II clinical study, Voges et al. treated recurrent GBM with HSV-1-TK gene-bearing cationic liposomal vector and systemic GCV and observed a therapeutic benefit in few of the patients. This study results demonstrated the feasibility and safety of this therapeutic strategy [33]. In another study, Nasu et al. conducted a phase I clinical trial for hormone-refractory prostate cancer. The treatment involved the administration of adenoviral vectors containing HSV-TK gene followed by GCV injection and there was a clear decrease of prostate-specific antigen levels. This confirms the safety profile and clinical response at the surrogate marker level [34]. Given the successful results,

many of the studies have been advanced to phase III. In these phase III trials, patients received either standard therapy (surgical resection and radiotherapy) or a combination like standard therapy and adjuvant gene therapy during surgery. It was concluded that the adjuvant treatment improved neither time to tumor progression (TTP) nor overall survival (OS) rate, although the feasibility and good biosafety profile of this gene therapy strategy were further supported. The failure of this specific protocol was due to the poor rate of HSV-TK gene delivery to tumor cells. In addition, the current mode of manual injection of vector-producing cells limits the distribution of these cells and the released replication-deficient RV vectors to the immediate vicinity. Further evaluation of the gene therapy strategy must incorporate refinements such as improved delivery of vectors and transgenes to the tumor cells, noninvasive in-vivo assessment of transduction rates, and improved delivery of the prodrug across the BBB and BTB to the transduced tumor cells.

2.2. Cytosine Deaminase and 5-Fluorocytosine (CD/5-FC) Suicide System

The cytosine deaminase (CD) is found in several bacteria and fungi, but not in mammalian cells. The CD gene has been cloned from E. coli predominantly [39]. The CD catalyzes the hydrolytic deamination of cytosine into uracil which involves the conversion of the non-toxic prodrug 5-FC to 5-Flurouridine (5FU). 5-FU then is transformed by cellular enzymes into potent pyrimidine antimetabolites (5-FdUMP, 5-FdUTP, 5-FUTP), leading to induced cell death. It was observed that this cell death is caused by the mechanisms of thymidylate synthase inhibition, formation of (5-FU) RNA, and the formation of (5-FU) DNA complexes [38].

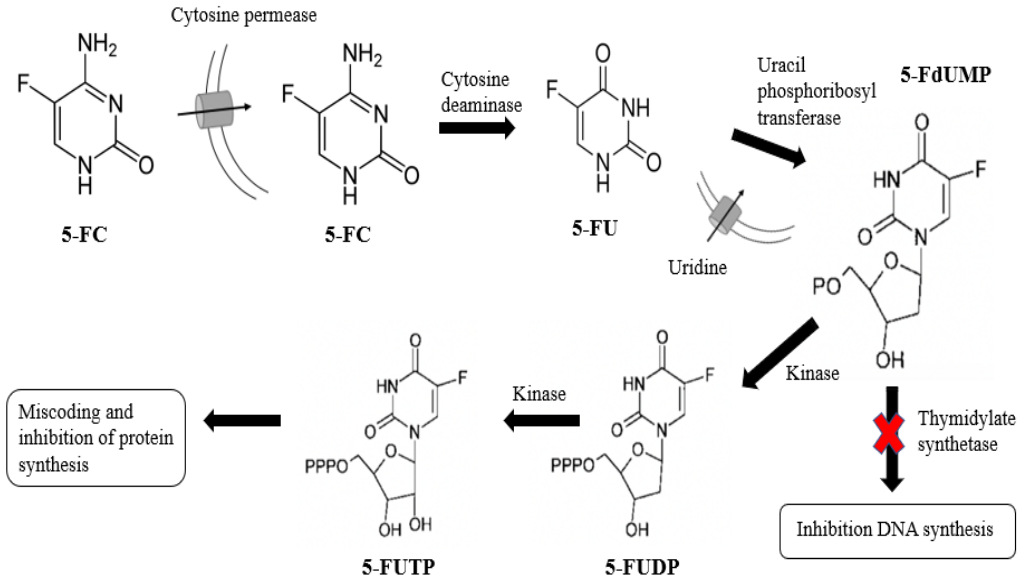


Figure 5 Mechanism of CD/5-FC Suicide System (Modified from BA McManus, 2009)

Apoptosis is primarily involved in the mechanism of CD/5-FC system, similar to HSV/TK-GCV [22]. Both the suicide systems induce cytotoxicity through a mitochondrial pathway but CD/5-FC involves down-regulation through the control of Bcl-2 [56] and the activation of heat shock protein (90- ß), contributing to tumor regression [57].

Though widely used in cancer chemotherapy, 5-FU is required in high doses for tumor response. The CD/5-FC system has been further improved in several studies by the inclusion of the UPRT gene which allows the conversion of 5-FU to 5-fluorouridine monophosphate; as a first step of its pathway to activation [41]. Surprisingly this CD-UPRT/5-FC suicide system was shown to be effective against 5-FU-resistant human primary cancer cells [42]. Finally, a bifunctional chimeric protein designated FCU1, combining the yeast enzymatic activities of CD and UPRT, was shown to highly increase CD activity [43]. In-vivo anti-tumor activity of the CD/5-FC combination

has been demonstrated in several animal models for indications such as fibrosarcomas [44], carcinomas [45–49], gliomas [50] and metastatic formations of different origin [51,52].

In one study, Nemunaitis et al. performed a pilot trial in refractory cancer patients, which involved intratumoral injection of TAPET-CD (an attenuated Salmonella bacterium expressing the E. coli CD gene). The authors reported that they observed an absence of adverse effects induced by TAPET-CD and the results demonstrated that the Salmonella bacterium can be utilized as a delivery vehicle of the CD gene to malignant tissue, and the delivered gene was found to be functional [54].

An oncolytic adenoviral vector containing a CD/HSV-1 TK fusion gene was applied in a phase I clinical trial of newly diagnosed, intermediate-to-high risk prostate cancer. It was found that the transgene expression persisted in the prostate for up to 3 weeks after the adenovirus injection. A combination of this therapeutic system with conventional dose and a three-dimensional conformal radiation therapy resulted in significant declines in prostate specific antigen level in all patients without noticeable toxicity [53].

2.3. Nitroreductase/CB1954 (NTR/CB1954) System

NTRs are predominantly used as GDEPT enzymes because when they metabolize the aromatic nitro groups to hydroxylamines, they generate a very large electronic change, providing an efficient switch that can be exploited to generate potent cytotoxins. While nitroreductase enzymes are widespread, nearly all the work using these in GDEPT has been with the nfsB gene product of E. Coli, an oxygen-insensitive flavin mononucleotide nitroreductase (NTR).

The antitumor activity of E. coli-NTR in combination with the prodrug CB1954 depends mainly on the reduction of the nitro groups to reactive N-hydroxylamine intermediates that are toxic in proliferating and nonproliferating cells [Figure 6]. This combination system was originally an attractive therapy for tumor treatment because the only human enzyme found to activate CB1954 was DT-diaphorase (also known as NAD(P)H Oxidoreductase). DT-diaphorase is a flavoprotein that catalyzes the two-electron reduction of quinones and is much less efficient in reducing CB1954 than the E. coli NTR [64]. The metabolites of CB1954 are potent alkylating agents which can kill both dividing and non-diving tumor cells. This is an advantage compared to some other GDEPT systems which target only one type of cells. The metabolites are highly cell-permeable which showed a strong bystander effect for killing the adjacent tumor cells [65]. The degree of bystander effect and the degree of transferred cytotoxicity correlates with the level of NTR enzyme expression.

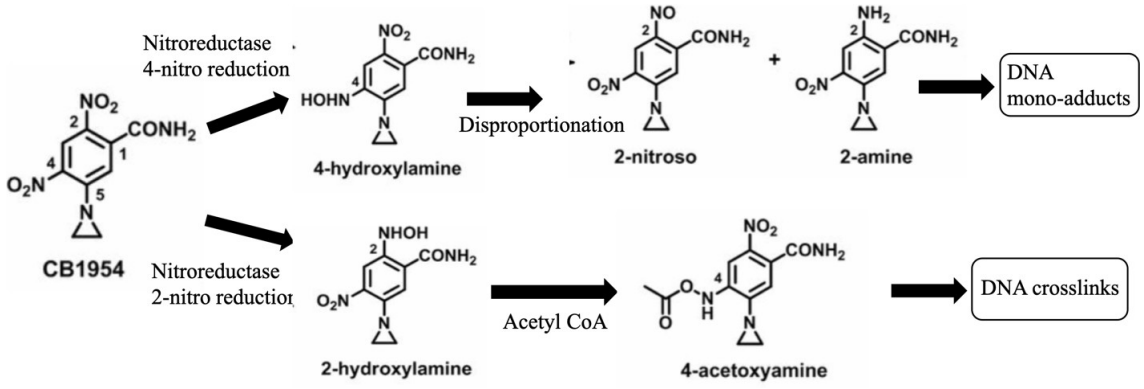


Figure 6 Mechanism of CB1954 in Tumor Cells (Modified from Mitchell, 2008).

Previous studies have confirmed many advantages of NTR/CB1954 as compared with the HSV-TK/GCV system. NTR/CB1954 suicide system is independent from the cell cycle, has higher ability to target both dividing and growth-arrested cancer cells, and induces potent bystander effect on the cell cycle. The bystander effect, in this context, is the death of non-transgenic cells, due to

indirect effects of treatment of neighboring transgenic cells, causing wider more spread of cell death rather than if transgenic cells alone were killed. Hence, to enhance the NTR/CB1954 system the key is an increased tissue penetration capacity of the prodrug, and the subsequent ability of the activated metabolites to spread to adjacent non-transduced cells. This system proved to be effective in few clinical trials for the treatment of liver and prostate cancers [66].

2.4. Cytochrome P450/Cyclophosphamide (CYP450/CPA) System

The physiological role of P450 enzymes is to deactivate compounds by oxidation. This feature is used to convert non-toxic prodrugs into active, tumor cell killing metabolites in GDEPT systems [Figure 7]. The advantages of P450-based GDEPT system include the feasibility of using human P450 genes to limit host immune response and its compatibility with existing anti-cancer drugs. CYP2B6 is primarily responsible for the hydroxylation of CPA while CYP3A4 metabolizes ifosfamide (IFA), an isomer of CPA. This metabolism yields 4-hydroxyl derivatives that further get converted to bioactive metabolite acrolein, and cytotoxic metabolite phosphoramide mustard. These metabolites create inter-strand crosslinks in the DNA which subsequently create strand breaks during DNA replication causing cell death.

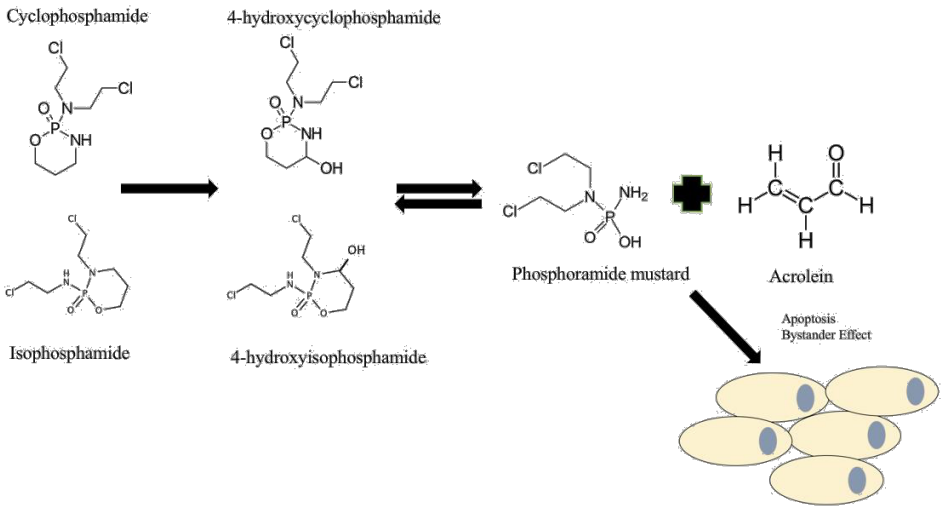


Figure 7 Mechanism of Phosphamides in Tumor Cells (Modified from Hedley et al., 2007)

Chloroacetaldehyde is another cytotoxic metabolite generated by CYP3A4 through N-dechloroethylation. This metabolite contributes to the anti-tumor therapeutic activity and severe neurotoxicity and urotoxicity. This pathway consumes only 10% of administered unlike up to 50% of the IFA [67]. CPA is mostly used for the treatment of lymphoma, leukemia, multiple myeloma, neuroblastoma, retinoblastoma, ovarian cancer, breast cancer, and endometrial cancer. IFA is commonly used for the treatment of soft tissues sarcomas, testicular, ovarian, and breast cancer.

This system has already been applied in various clinical trials. Studies have concluded that the phosphoramide mustard is unable to cross the cell membranes for producing an effective local tumor concentration and unable to induce strong bystander effect if the drug is activated in the liver [67]. A few strategies have been successfully used to enhance the effectiveness of P450 GDEPT for cancer treatment. For example, a P450 reductase gene has been co-expressed to facilitate P450-dependent prodrug activation and cytotoxicity [68]. Experiments have been conducted using anti-

apoptotic factors to delay tumor cell death and prolong bystander effect [69]. A phase 1 clinical trial was performed utilizing the MetXia-P450 recombinant retrovirus vector carrying CYP2B6 gene in combination with oral cyclophosphamide to treat advanced breast cancer or melanoma. The initial trial results not only confirmed the safety of this approach but also demonstrated consistent levels of gene expression in the cancer cells.

2.5. Carboxypeptidase G2/4-[(2-mesyloxylethyl) (2-chloroethyl) amino] Benzoyl-l-glutamic Acid (CPG2/CMDA) System

The gene for bacterial enzyme CPG2 was expressed internally in mammalian cells and was found that the mammalian expressed CPG2 has kinetic properties indistinguishable from bacterially expressed CPG2. The natural substrate of CPG2 is folic acid, but it is capable of hydrolyzing glutamate which allows its usage in activation of synthetic prodrugs, in GDEPT and ADEPT. CPG2 catalyzes the hydrolytic activation of CMDA [Figure 8]. This reaction generates glutamic acid and the parent cytotoxins, including BANM, ANM and PNM.

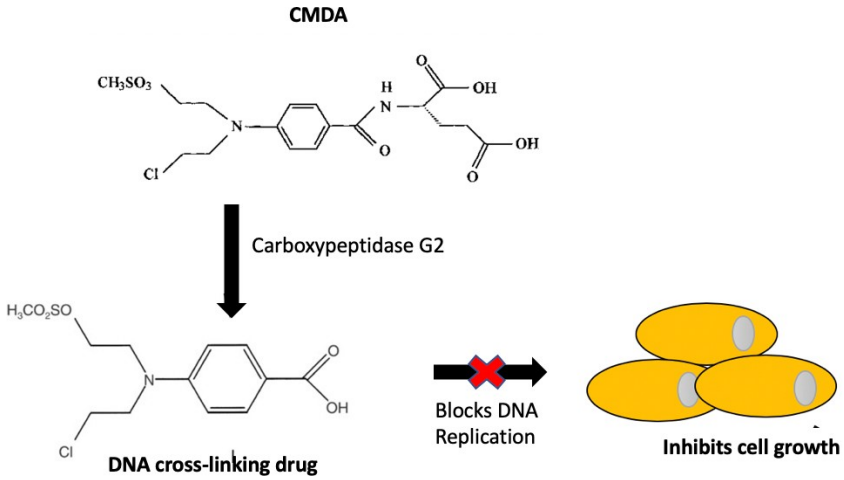


Figure 8 Mechanism of CMDA in Tumor Cells (Modified from Y Jamin, 2014)

The success of CPG2-based GDEPT primarily depends on the timing of injection of the prodrug following expression of the transgene and also depends on the generation of enough concentration of CPG2 in the tumor [70]. To monitor the prodrug- activating enzyme activity and its biodistribution, non-invasive imaging strategies were developed, so as to guide successful translation of this therapeutic approach to the clinic [71].

Table 1 summarizes the most commonly explored systems for suicide gene therapy with their origin, the prodrug used, the cytotoxic compounds, the mechanism of action, whether bystander effects plays an important role and the type of cancer the systems have been tested on.

Table 1 Summary of GDEPT Systems with Mechanisms of Action

Enzyme	Origin	Prodrug	Toxic Metabolite	Mechanism of Action (MOA)	Bystander Effect	Cancer Type
Thymidine Kinase (TK)	Herpes Simplex Virus (HSV)	Ganciclovir (GCV)	Ganciclovir (GCV)-Triphosphate	Blocks DNA synthesis, S and G2 phase arrest, Mitochondrial damage	High when GJIC exists	Malignant Glioma, Pancreatic, Adenocarcinoma
Cytosine Deaminase (CD)	E. coli, Yeast	5-Flurocytosine (5-FC)	5-Flurouracil (5-FU)	Blocks DNA and RNA synthesis	High and independent of GJIC	Colon, Glioma, Pancreatic
Cytochrome 450 (CYP450)	Human, Rat, Rabbit	Cyclophosphamide	Phosphoramide mustard, Acrolein	DNA inter strand crosslinker	Medium and independent of GJIC	Pancreatic
Nitroreductase (NTR)	E. Coli	CB1954	2,4-hydroxylamine	DNA inter strand crosslinker	Very high and independent of GJIC	Prostate, Liver
Carboxypeptidase G2 (CPG2)	Pseudomonas	Nitrogen Mustard	N, N-2(-chloroethyl) (2-mesyloxyethyl aminobenzoic acid	DNA inter strand crosslinker	High and independent of GJIC	Ovarian, Adenocarcinoma, Colon Carcinoma

CHAPTER 3 - SUICIDE GENE DELIVERY SYSTEMS

An effective gene delivery to tumor cells is an essential component for successful suicide gene therapy. Many different types of gene delivery systems have been explored for delivery of suicide gene in GDEPT and it was observed that the intratumor injection was employed to maximize the delivery efficiency. The following are a brief description on characteristics of each type of gene delivery system, either viral or non-viral vectors, used in suicide gene therapy.

3.1. Viral vectors

As viruses have been evolved from natural mechanisms to deliver their genomes into cells, they are excellent vectors to deliver foreign DNA. They are extremely effective in transferring genes and are the most advanced in development. These viral vectors are designed by replacing non-essential genes (involved in viral replication or pathogenic protein production) with foreign therapeutic genes. As a part of their infection process, they bind to target cells and introduce their genetic materials into the host cell. Efficiency and specificity in entering cells and expressing the carried genes is what makes them useful in gene transfer, especially in-vivo.

The following is a brief description on major types of viral vectors examined for suicide gene therapy studies.

3.1.1. Adenoviral vectors

These are derived from adenoviruses that usually cause mild respiratory, digestive and ocular infection in humans. Over 100 serotypes of adenoviruses have been identified so far. These

viruses are icosahedrons, consisting exclusively of proteins and DNA. The protein shell is composed of subunits called capsomeres, of which majority are hexons and few are pentons. Each penton contains a base, which forms a part of the capsid and a projecting fiber whose length varies among different serotypes. The projecting fiber interacts with a cellular receptor protein called CAR [73]. Efficient entry of adenovirus is facilitated with the presence of CAR and a secondary interaction between the penton base protein and integrins on the cell surface [74, 75]. They pose a very high transduction efficiency with very few tendencies for viral shedding and latent infection. They can easily be produced commercially in large quantities, and high titers are easily achievable (10^{11} to 10^{12} particles per ml). They not only can carry prodrug genes but also others.

The mostly used adenoviral vectors in gene transfer studies are from the human type 5 adenovirus. The first generation of adenoviral vectors consists of viral genome with replacement of E1 and E2 genes (involved in early steps of viral DNA replication) with a therapeutic gene. The latest generation of adenoviral vectors is the gutless adenovirus (with deletion of the entire viral DNA sequence) which has better safety profile, and long-term sustained gene expression [76].

3.1.2. Retroviral vectors

Retroviruses are enveloped virions whose genome comprises of three genes termed gag, pol, and env. They are flanked by 2 elements required for integration called long terminal repeats. Murine leukemia virus (MLV) and its derivatives were the first form of retroviral vectors to be used in gene therapy [77-79] because the retroviral life cycle was well studied and the technology to generate recombinant vectors devoid of replicating helper virus was well developed [80].

Retroviral vectors are particularly attractive because the transgenes they carry integrate into the target genome and can potentially provide permanent gene expression.

Like all viruses, retroviruses have a specific requirement for interaction with a cell surface receptor for infection. Retrovirus main choice is a phosphate transporter, whose function is unrelated to their roles in virus infection. Retroviral vectors can accept up to 8 kb of gene fragment, which replaces all viral genes, and retains LTRs and a packaging sequence. Only if target cells undergo cell division, the integration of proviral DNA proceeds shortly after the infection. This characteristic has made retroviruses the vehicle of choice for targeting of tumors, because the surrounding normal tissue is inactive. But there are chances of insertional mutagenesis risk due to the integration of the provirus in the host genome. However, recent developments in self inactivating retroviral vectors [81] have made retroviruses potential candidates for the incorporation of tissue specific promoters. An attractive feature of retroviruses is that they essentially produce the desired protein products hence there is no induction of host immunity from the production of additional proteins. The problem with in-vivo use of these vectors is their low transduction efficiency. Moreover, cells transduced with retroviruses appear to lose expression of transgene through loss of proviral sequence or methylation of the integrated DNA.

3.1.3. Lentiviral vectors

Lentiviruses are derived from HIV which is an enveloped virus that contains ssRNA genome. When the virus carrying a reverse transcriptase enzyme enters the cytoplasm, it transcribes RNA into DNA which then integrates permanently into genome of the target cells. Researchers have removed the infectious parts of the virus and added other parts from different

viruses such as cytomegalovirus with the help of genetic engineering, generating a highly modified and replication deficient lentiviral vector. Lentiviral vector has the advantage of being relatively safe, and have variable specificity to different types of cells, and also have efficient transduction of both dividing and non-dividing cells.

3.1.4. Adeno-associated viral vectors

AAVs are defective members of the parvovirus family. The name defective is because AAVs requires the presence of other helper viruses (like adenoviruses or HSV) to produce lytic infections unlike latent infection produced when they alone are integrated into the host genome. AAV genome is encapsulated as ssDNA and the vector is made by replacing the Cap and Rep genes with a transgene of interest. AAV enters the cell via a heparan sulfate proteoglycan [96]. AAV type 2 has been the most commonly used vector for gene therapy studies. Initially, the productions of the vector were laborious as they required purification of AAV particles from wild type adeno viruses but more progress has been made recently that has allowed the production of AAV vector without using a wild type adenovirus, and also increases viral titers [94, 95]. This vector has been successfully used in gene therapy due to its broad tropism capable of transducing a wide range of cell types in various organisms. Disease free properties of AAV and the persistence of gene expression it generates make AAV a very promising gene therapy tool.

3.1.5. Herpes-Simplex Virus

HSV causes mucocutaneous infections including genital herpes, ocular infection, and CNS infection. It is a non-enveloped virus with a dsRNA genome and is generally considered benign, with good safety records and is less expensive to be produced commercially.

A variety of viral vectors with different characteristics have been used for gene delivery. The choice of vector depends strongly on the strategy in use, and on desired attributes for achieving a positive outcome. This includes characteristics such as integrating vs non-integrating vectors, size of the transgene, capacity of the vector, ability of the vector to target certain cell types, and feasibility of having the required number of viral particles synthesized for in-vivo delivery.

3.2. Non-viral vectors

In addition to viral vectors, the non-viral vectors have also been considered for gene delivery. Major types of non-viral vectors studied include the cationic liposomes and cationic polymers. Being cationic, these delivery systems interact with the negatively charged DNA and form complexes which facilitate intracellular gene delivery via endocytosis. Being synthetic, the major advantages of non-viral vectors are their safety and ease of large-scale production.

3.2.1. Cationic liposomes

Cationic liposomes are made of 1 or more lipids such as DOTAP, DOPE and cholesterol. During travel from the site of injection to intended target cells, cationic liposomes form stable complexes with polyanionic DNA or RNA protecting their degradation from nucleases. The complexes eventually interact with target cells leading to the entry of DNA and thus expression of

the transgene. Chemical modification on lipids, liposomes, or complexes may result in difference in gene delivery efficiency, target specificity and alter the stability in blood. Preclinically, cationic liposomes have been explored for both DNA and siRNA delivery. Major drawbacks of this gene delivery system are dose-dependent toxicity, hepatotoxicity, and pulmonary inflammation for airway delivery while rapid clearance from the blood (when intravenously injected), and complement activation are additional challenges.

3.2.2. Cationic polymers

Polymers are synthetic gene delivery vectors that has the ability to deliver genetic material to target cells. PEI, PLL, PDMAEMA and chitosan are some of the widely studied cationic polymers. These polymers neutralize the negative charges on plasmid DNA resulting in the formation of nanoscale polymer-DNA complexes (polyplexes). But there are a variety of complex biological barriers in the extracellular body fluid, extracellular matrix, and at the cellular level (such as cellular entry, escape from endo-lysosomal vesicles, cytoplasmic trafficking, DNA unpackaging, and nuclear translocation of the therapeutic DNA, transcription, and translation) that limit the transgene delivery efficiency and product level of transgene. High-throughput screening techniques have been employed in the pharmaceutical industry for facilitating the rapid evaluation of polymer structures for optimal gene delivery.

CHAPTER 4 - BYSTANDER EFFECTS IN SUICIDE GENE THERAPY

An interesting phenomenon observed in suicide gene therapy studies was a better expected response rate when suicide gene was delivered only to a small percentage of tumor cells. The results show that cytotoxic effects were not only seen in tumor cells expressing the suicide gene but also in those neighboring cells without any suicide gene. This is because the converted cytotoxic drug molecules can pass through cell membrane and reach the neighboring cells. This phenomenon is called bystander effect. With this bystander effect, the therapeutic effects spread beyond the transfected tumor cells causing regression of distant tumor cells. There are currently 5 mechanisms investigated on bystander effect: a) release of cytotoxic drug by the targeted cells, b) passage through gap junctions, c) passive transportation, d) stimulation of local microenvironment, and e) endocytosis of apoptotic vesicles.

Among the above-mentioned mechanisms, gap junction-mediated intercellular communication (GJIC) appears to be the most important mechanism observed in several suicide gene/pro-drug systems [97-101]. GJIC is a natural function of cells to maintain homeostasis in organs as it involves transfer of ions, nucleotides, small regulatory molecules as well as drugs or their metabolites between adjacent cells [104]. Evidence suggests through these communications, the gap junctions allow a passive diffusion of the cytotoxic drug molecules to neighboring cells, leading to apoptosis of cells with or without suicide gene expression and enabling the drug to kill a greater cell population.

The membrane structures of gap junction consist of connexon, which is an assembly of proteins called connexins. These connexins form a gap junction between the cytoplasm of two

adjacent cells. In general, tumor cells have reduced intercellular communication as compared to normal cells [107, 108]. Connexin transfection of tumor cells leads to decreased rate of proliferation, increased differentiation, and reversal of cell transformation phenotype [102, 103].

Connexin 43 has been identified as one of the major connexins in breast tissue which is often lost or reduced in cancer [104]. Many studies have shown that over expression of connexin 43 gene by gene transfer results in increased GJIC, leading to an increased bystander effect. [97, 98, 105]. Studies also suggest that the bystander effect is precisely the reason that a whole tumor mass can be eliminated without having the gene to be expressed in every cell. Coupling transfer of connexin 43 cDNA to the suicide gene therapy would be useful for enhancing the bystander effects and antitumor activity. Chemical induction of connexin 43 expression might have a more potent effect, since a greater proportion of the cancer cell population can be reached by small chemicals as compared to gene delivery.

Bystander effects are broadly classified into 2 categories depending on the proximity between cells with and without suicide gene - the local bystander effects and distant bystander effects. Local bystander effect does not require cell-to-cell contact unlike the distant bystander effect, and drug diffusion between cells occurs through non-facilitated diffusion [113]. The CD/5-FU system shows stronger local bystander effect than the HSV-TK/GCV system as the 5-FU diffuses efficiently within the tumor cells and does not require cell to cell contact. The GCV-triphosphate mediated bystander effect uses different mechanisms. One mechanism that the HSV-TK/GCV uses is the local inflammation and devascularization, which probably enhance the vascular permeability and the cytotoxic drug diffuses more efficiently [115]. The distant tumor regression effect was initially attributed to the stimulation of immune system.

It was first demonstrated that the bystander effect requires cell-to-cell contact, which suggests the passage of toxic GCV metabolites from TK-expressing cells to unmodified tumor cells. The requirement of gap junctions was then observed after comparing the bystander effect in tumor cell lines, transfected or not with connexin genes, which was also revealed in human primary malignant glioma cells in culture [118, 119]. In parallel, using flow cytometric and electron microscopic analysis, Freeman et al. demonstrated that the bystander effect can also be mediated via apoptotic bodies generated from dying TK-expressing cells and phagocytosed by unmodified neighboring cells [110]. More recently, the expression of E-cadherin, which is involved in the formation and function of gap junctions, was shown to correlate strongly with the TK/GCV bystander effect. The co-expression of TK and E-cadherin genes mediated by an adenoviral vector improved TK/GCV cytotoxicity and triggered a potent anti-tumor effect, superior to the one generated by an adenoviral vector expressing only TK. Moreover, the increased expression of E-cadherin was found to be associated to a decrease in the bcl-2 content, which suggests that a high E-cadherin content improved TK/GCV therapy by both enhancing the bystander effect and facilitating apoptosis induction [113].

Regarding distant bystander effect, regression of untransduced tumor cells growing at a distant site from transduced tumors was achieved in HSV-TK/GCV suicide gene therapy. To explain the TK-related distant anti-tumor effect, several hypotheses have been proposed. The involvement of the immune system was proposed in most studies, based on the presence of T lymphocyte infiltrates within the tumors [120,121–124]. However, a distant bystander effect was also detected in immunodeficient SCID mice, which suggests that alternative mechanisms may be operating, such as the release of a soluble factor that might contribute to the observed distant anti-tumor effect. For CD/5-FC suicide system, a distant bystander effect was observed

after injection of CD-expressing tumor cells; or after CD gene delivery via naked DNA injection. Such an immune effect acting on pre-established wild-type tumors was dependent on both CD4+ and CD8+ lymphocytes as well as on natural killer cells [125-128].

Results accumulated so far support the notion that bystander effect is the most critical and essential fact for suicide gene therapy.

CHAPTER 5 - SUICIDE GENE THERAPY IN COMBINATION WITH OTHER THERAPEUTIC STRATEGIES

The combination of CD/5-FC and HSV-TK/GCV suicide systems was studied and it was observed that they enhanced the anti-tumor activity in both in-vitro and in-vivo. It was concluded that the sequential treatment was more successful than the simultaneous addition. This is because the CD/5-FC-mediated reduction of dTTP resulted in a significant decrease of dGTP, which is the endogenous competitor of GCV-triphosphate.

Suicide gene therapy was also shown to synergistically act with other classical anti-cancer treatments. Radiotherapy can further be enhanced using replication competent vectors which was demonstrated by several clinical trials. The therapeutic benefits and safety of this approach was well evident [129-132]. Oncolytic viral vectors along with a suicide gene were also successfully used in several non-clinical studies [133–137].

The combination of conventional chemotherapy and suicide gene therapy with was also tested and it showed a great efficacy, both in-vitro and in-vivo [138–142]. But the combination of suicide gene therapy with RNAi-based therapy was found to be more efficient. Various combined strategies involved co-transfer of a suicide gene and an immune-stimulatory gene (such as cytokine and chemokine genes) as suicide gene therapy can trigger an immune response. It was shown that there was increased cell apoptosis when IL-2 was combined with HSV-TK genes [144].

CHAPTER 6 - FUTURE PERSPECTIVES

Conventional chemotherapy aims inhibition of the critical enzyme activity to block DNA replication, mRNA transcription, translation, or directly causing DNA damages to stop the proliferation of cancer cells. A significant drawback of conventional therapy is the toxicity associated with normal cells with a property of active proliferation including those in bone marrow, hair follicles and the gastrointestinal tract. Therefore, a targeted therapy seems needed to limit cytotoxicity to tumor cell only.

The prodrug approach or suicide gene therapy has been developed to minimize the toxic effect of conventional therapy by limiting cytotoxic drugs only to cancer cells. It was achieved by tumor-specific delivery of an enzyme-coding gene producing a gene product that will catalyze an intracellular conversion of a non-toxic compound to a cytotoxic drug molecule, leading to apoptosis and tumor elimination. Results accumulated so far from preclinical and clinical studies appear to validate this concept and prove that prodrug therapy is a viable approach for the treatment of cancer. However, like many therapies currently available, continuous efforts are needed to overcome the remaining bottlenecks of the suicide gene therapy.

The catalytic activity of an enzyme is critical to achieving high drug concentration in cancer cells, essential to induce cell damage and apoptosis, and to spread to neighboring cells for bystander effect. Research to optimize the enzyme activity for high specificity and efficiency would make prodrug conversion more effective. In fact, the mutagenesis-based strategy has

been used to obtain more efficient activation of a given prodrug or to adapt the active site so that it binds better to prodrugs that are not substrates for endogenous enzymes.

Another major challenge in suicide gene therapy is the effective activation of specific prodrugs. The prodrugs could also be redesigned to create better substrates for the enzymes, to maximize drug release, or to allow the active drug to accumulate more readily in tumor cells. It will also be useful to investigate how different prodrug systems synergize with each other or with other strategies of cancer treatments (e.g. combination therapy).

Another significant bottleneck for suicide gene therapy is lack of a safe and effective vector for tumor-specific gene delivery. Although close to 100% gene delivery efficiency has been reported in cell culture, the highest efficiency in tumor gene delivery in-vivo is less than 10%, making complete elimination of tumor cells less likely, even with bystander effect. The effort in developing an improved gene delivery system, either viral or nonviral represents a priority. Breakthrough in achieving high level of gene delivery to tumor cells could make suicide gene therapy a reality for cancer treatment. Minimizing immunogenicity and insertional mutagenesis associated with current viral vectors is another critical area for research. The ongoing research to improve gene delivery efficiency of nonviral vectors may lead to an elimination of viral-vector approach for gene delivery. An artificial gene delivery system is preferred over viral vectors due to its lack of safety concern and ease for large scale preparation.

Unconventional/non-viral biological gene delivery vehicles like bacteria, bacteriophages, virus-like particles, erythrocyte ghosts, and exosomes have been the most extensively studied for cancer gene therapy. Nanoparticle-based delivery systems have also emerged as potential gene carriers due to their unique properties of nano-scale matter. These

vectors can enhance tumor accumulation of the carried biologically active agent due to the EPR effect. EPR is enhanced permeability and retention effect that is a result of a combination of increased permeability of tumor blood vessels and a decreased rate of clearance within the tumor. Consequently, after intravenous administration the nanocarriers passively accumulate in solid tumors. These kinds of vectors are presently used more often for siRNA than for plasmid DNA delivery.

Other limitations of suicide gene therapy include limited spatial distribution of gene transfer vectors, inability to reach cells deep in a solid tumor and target metastasized tumor cells in different locations. When using viral vectors, there are limited tumor targeting capabilities as there is a possibility of them being neutralization by immune system. When using a non-viral vector, it can get toxic to surrounding tissues and the delivery of genetic material can be inefficient.

Patient studies with suicide gene therapy are still limited and every effort towards this treatment modality must be welcomed. Several new pathways as targeted suicide gene therapy are under investigation along with new promoters for better gene expression. Efficient suicide therapy depends not only on the characteristics of the targeted tissue, but the organ affected by the tumor. A study should be designed considering both the parameters. Currently, there are several delivery and effector systems available for suicide gene therapy. Each of them demonstrates effectiveness against specific cancer type. Combining different systems has demonstrated enhanced antitumor efficiency. Combination with interventional techniques has also shown enhanced antitumor activity in less time than a single modality therapy in tumor models involving extensive tumor vascularization. It is highly expected that continuous effort and additional research will lead to better strategies and approaches that will overcome the

limitations of current suicide gene therapy approaches. It is highly anticipated that the concept of targeted cancer therapy using suicide gene will lead to a better treatment for cancers.

Table 2 Summary of Suicide Gene Therapy

ADVANTAGES
• Cancerous cells or rapidly growing cells are more susceptible to impaired DNA synthesis, which in turn reduces the growth rate of these cells • Gene therapy using HSV-TK/GCV could kill neighboring tumor cells • Tumor cells which are chemo resistant or drug resistant in nature can also be treated effectively using suicide gene therapy.
DISADVANTAGES
• Suicide gene therapy using HSV-TK technique enhanced growth, invasiveness, and chemotherapy resistance of tumor cells. • Clinical efficacy of suicide gene therapy is also limited by the low affinity of GCV towards HSV-TK • GCV inadequately transported into the cell and its phosphorylation level is also low, therefore GCV may not be the best substrate for HSV-TK • Effect and uptake of GCV is also varies depending upon the type of tumor cells • GCV uptake by the cell is also dependent on the expression of HSV-TK in the cell, that is if the expression of HSV-TK is low in the cell then the uptake of GCV will also decrease considerably

FUTURE

- Cancer can be treated effectively by combining traditional chemotherapy, radiotherapy method along with suicide gene therapy technique.
- Combining prodrug therapy along with suicide gene therapy is also effective in treating tumor and cancer
- Combining two or more genes can be used in suicide gene therapy
- HSV-TK when combined with other genes also showed increased efficacy in killing tumor cell lines. Hence the technique can be used in suicide gene therapy to treat patients with cancer
- HSV-TK genes when combined with other gene related to the immune system of human may be used in suicide gene technique
- Interesting characteristic of this technique known as suicide gene therapy is that different genes can be combined with HSV-TK/GCV therapy to get maximum efficiency to treat different types of cancer
- By considering all these points one can agree that suicide gene therapy is a promising solution to kill tumor or cancer cells.

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