SUICIDE GENE THERAPY OF CANCER

AN EXAMINATION OF CYTOSINE DEAMINASE PLUS 5-FLUOROCYTOSINE SUICIDE GENE THERAPY IN COMBINATION WITH CISPLATIN CHEMOTHERAPY FOR THE TREATMENT OF CANCER

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ABSTRACT

Cancer is a disease characterized by complexity and unpredictability. Consequently, its treatment is difficult and all too often unsuccessful. Almost all cancers are treated with some combination of the traditional anti-cancer armamentarium: surgery, chemotherapy, and radiotherapy. Recently, however, gene therapy has emerged as a promising addition to this existing repertoire. Its application as a single agent, or in combination with other anti-cancer treatments is proving successful in both pre-clinical and clinical settings. In this work I have investigated the combination of a conventional chemotherapy drug, cisplatin, with a type of cancer gene therapy known as cytosine deaminase + 5-fluorocytosine suicide gene therapy.

Suicide gene therapy is the intracellular conversion of non-toxic prodrug to its active form by a metabolic enzyme of non-mammalian origin. There are many established enzyme/prodrug combinations, but here the bacterial enzyme cytosine deaminase (CDA) was used to convert inert 5-fluorocytosine (5FC) to highly toxic 5-fluorouracil (5FU). Of the various vector systems for therapeutic gene delivery, adenoviral (Ad) vectors have proven particularly suitable for application to cancer. This work used a first generation adenovirus type 5 vector expressing the enzyme cytosine deaminase (AdCDA) cloned from *E. coli*.

The combination of AdCDA/5FC with cisplatin was chosen because the combination of 5FU and cisplatin, both of which are used extensively in cancer treatment, has proven effective clinically and demonstrates synergy *in vitro*. This combination was evaluated in murine mammary carcinoma MT1A2 cells, human colorectal carcinoma HT29 cells, HT29p14 cells, the photofrin resistant sub-line of HT29 cells, and murine melanoma B16/F10 cells. The classical clonogenic assay was used to evaluate this combination treatment since it provides an accurate indication of the effectiveness a cancer treatment will have *in vivo*.

AdCDA infected MT1A2, HT29, and HT29p14 cell lines exhibited a dose response to increasing concentrations of 5FC that was significantly different from control vector infected cells. Similarly, uninfected cells demonstrated a dose response to increasing concentrations of cisplatin. The effect of the combination on clonogenic survival, administered in the sequence of a 48 h exposure to 5FC followed by 1 h exposure to cisplatin, was greater than additive compared to the effect of the two treatments alone.

F10 cells exhibited a dose response to increasing concentrations of cisplatin. However, it could not be shown reproducibly that AdCDA infected F10 cells exhibited a dose response to 5FC that differed significantly from control vector infected cells. Work with the F10 cells was inconclusive regarding the combination treatment, but it rendered information regarding the sensitivity of these cells to what is hypothesized to be an unidentified component present in some preparations of 5FC.

Evaluation of this treatment *in vivo*, using both murine and human tumor cell lines, will further define the potential of AdCDA/5FC + cisplatin as a clinically relevant cancer treatment.

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This thesis epitomized collaboration, and there are many people to whom I wish to express my thanks.

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This thesis is dedicated to my parents and my brother.

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CHAPTER 1

INTRODUCTION

Cancer

Cancer is characterized by uncontrolled growth of genetically aberrant cells. It begins with genetic change in a single cell, conferring upon it the ability to grow in this abnormal manner. This transformed cell then proliferates to form a tumour. The tumour continues to grow and may invade the normal tissue surrounding it. As it increases in size, it induces development of vasculature to provide blood flow to support its growing mass. This process is called angiogenesis. Invasion into local tissue and establishment of neovasculature allow tumour cells to metastasize. Metastatic tumour cells are essentially seeds, from which other malignant tumours will develop at sites elsewhere in the body (Cotran *et al*, 1999).

The biology of cancer is characterized by extreme diversity and complexity. The genetic plasticity and instability of tumour cells have made treating the disease as difficult as it is to understand or predict it. Traditional cancer therapies, such as surgical resection, chemotherapy, radiotherapy and even biological therapy, are by no means ineffective. The ever-increasing number of cancer survivors is a testament to the curative potential these treatments hold. However, there are shortcomings to these therapies. They are toxic and can be carcinogenic, they may lack specificity for the tumour, or the tumour may become refractory to the therapy. Or it may be that the treatment causes regression, but not complete elimination, of the tumour. These limitations are well-established and often pose a significant barrier to increased survival, quality of life, and rates of cure. A promising alternative to these highly toxic and often potentially carcinogenic therapies is gene therapy.

Gene Therapy of Cancer

Cancer gene therapy is the use of therapeutic transgenes for the treatment of cancer. The transgene is delivered to the patient using a gene delivery vector. Expression of the transgene within the patient produces a therapeutic protein that mediates some anti-tumour effect. It is an approach to cancer treatment that is emerging in tandem with developments in other areas of science. As descriptions of the immunological, cellular, and molecular biology of cancer evolve, so too do concepts of how to target the disease more specifically and more efficiently than traditional radiotherapeutic and chemotherapeutic approaches. Similarly, innovations in techniques of genetic manipulation are better facilitating the transfer of gene therapy strategies from theory into practice.

In the development of gene therapy, for any disease, there are several critical considerations: what is the desired therapeutic effect; how will the gene be delivered and what will be the overall effect of vector administration. Development of methods for gene delivery is a highly active field of investigation. Basic delivery strategies include both non-viral vectors (naked or liposome encapsulated DNA) and viral vectors (adeno-associated viruses, adenoviruses, herpes viruses, lentiviruses, poxviruses, and retroviruses) (Rochlitz, 2001). Given the availability of a wide variety of these strategies, each must be evaluated for the appropriateness of its application to a particular disease. For example, inherited genetic diseases, such as cystic fibrosis or haemophilia, require a therapeutic effect sustained throughout the life of the individual. Cancer, on the other hand, is a disease which generally requires treatment for a distinct, finite period of time. Thus, it is important that the choice of cancer gene therapy vector reflect treatment requirements that are specific to cancer.

Of the gene therapy vectors, retroviral and adenoviral (Ad) vectors have been developed extensively for use in cancer gene therapy. It is likely, however, that Ad vectors may be more suitable for cancer gene therapy. Retrovirus vectors integrate into the host genome while Ad vectors persist episomally, in the nucleus. This is an important safety consideration since the retroviral vector could insert into and disrupt a cellular gene, such as a tumour suppressor or oncogenes (Kurian *et al.*, 2000). This is important also because the transience of gene expression associated with a non-integrating vector is desirable for cancer gene therapy, as described above.

In humans Ad is mildly pathogenic. It causes infections of the upper-respiratory and gastrointestinal tracts which are characterized by symptoms similar to those of the flu (Hitt & Graham, 2000). There have been approximately 50 serotypes of Ad isolated from humans to date (Hierholzer *et al.*, 1988). Of these, serotypes 2, 5, 7, and 12 have been best characterized and primarily types 2 and 5 have been developed as gene therapy vectors (Hitt *et al.*, 1997).

The biology of Ad supports its suitability for therapeutic gene delivery (reviewed in depth, Hitt, Parks, & Graham, 1999). It infects both dividing and quiescent cells from a wide variety of mammalian species, is easily manipulated and can be grown to high titer (Bramson *et al.*, 1995). Thus the design, production, and testing of Ad vectors *in vitro* and *in vivo* can be accomplished without the overwhelming technical complications that are sometimes associated with vector propagation. Disadvantages to the use of Ad vectors in gene therapy include transience of gene expression, limited cloning capacity, and the host anti-Ad immune response. Of most concern for the clinical application of Ad vectors is the host immune response. Viral proteins as well as the transgene itself are immunogenic and can stimulate cellular and humoral immune responses in humans (Yang *et al.*, 1994) (Christ *et al.*, 1997). Since most humans have been exposed to Ad, levels of transgene expression following therapeutic vector administration would be compromised because of pre-established immunity. Also, the anti-Ad immune response may pose a significant health risk to the host. Consequently, a major component of Ad vector development is focused on modifying the vector to reduce its overall immunogenicity.

Modifications to the vector genome have produced a number of widely used Ad vector systems. Currently, first generation Ad vectors are used most extensively in cancer gene therapy (Hitt *et al.*, 1999). First generation vectors have had deletions in the early 1 (E1) and early 3 (E3) regions of the adenoviral genome. E1 genes are responsible for viral replication and their deletion renders the virus replication deficient. E3 genes are used by the virus to evade the host immune response and their deletion, in addition to deletion of the E1 genes, provides space within the Ad genome for insertion of the therapeutic gene, which can be up to approximately 8.3 kb in length (Bett *et al.*, 1994). First generation Ad vectors are propagated *in vitro* in 293 human embryonic kidney-derived cells (Graham *et*

al., 1977). 293 cells provide E1 functions *in trans* but do not substitute for the lost E3 genes, since they are not required for viral replication *in vitro* (Hitt *et al.*, 1999).

Cancer gene therapy is a highly active, intense area of basic scientific and medical research. While many systems remain in pre-clinical stages of development, some have progressed to human clinical trials. Currently there are human cancer gene therapy trials investigating cytokine immunomodulation, restoration of tumour suppressor gene function, suppression of activated oncogenes, and induction of drug sensitivity within tumour cells, also known as *suicide gene therapy* (Lattime & Gerson, 1999).

Suicide Gene Therapy of Cancer

Suicide gene therapy is a genetic negative-selection technique whereby a novel gene is delivered to a population of cells where it mediates the intracellular conversion of a non-toxic prodrug to an active, cytotoxic metabolite. Suicide gene therapy is also referred to as gene-directed enzyme/prodrug therapy (GDEPT), virus-directed enzyme/prodrug therapy (VDEPT) (Springer & Niculescu-Duvaz, 2000), or enzyme/prodrug therapy (EPT).

In suicide gene therapy the suicide gene is a metabolic enzyme that converts an inactive prodrug to its active, cytotoxic form (Mullen, 1994). Suicide genes are usually viral or bacterial in origin and do not have a mammalian homologue. However if a homologue does exist, it is minimally active and converts insignificant levels of the prodrug compared to the suicide gene itself (Morris *et al*, 1999). Thus, significant conversion of the prodrug to the active drug occurs only in those cells targeted to express the transgene.

The choice of enzyme/prodrug combinations with which to develop suicide gene therapy strategies has been influenced by currently available antiviral and antimicrobial agents. The treatment of infectious disease is a prototypical negative selection system, the aim of which is to eliminate the pathogen while sustaining minimal damage to the tissues of the patient. The antimicrobial drug is toxic only to the pathogen because tissues of the host do not express the enzyme that catalyzes conversion of the prodrug. Suicide gene therapy uses these same combinations of enzyme and prodrug to facilitate elimination of an unwanted population of cells from a patient's body. First, the target tissue is transduced with the gene for the converting enzyme. Once expressed, it converts the prodrug to a

cytotoxic drug and thus eliminates the target tissue. An additional benefit to using existing treatments as a template for the development of suicide gene therapy is that one component of the combination is a substance already approved for use in humans. Therefore, a wealth of basic biochemical, safety and dosing data is immediately available for consideration in development of the suicide gene therapy strategy. This is invaluable, given the significant technical and regulatory hurdles already facing the transfer of any gene-based therapy into humans.

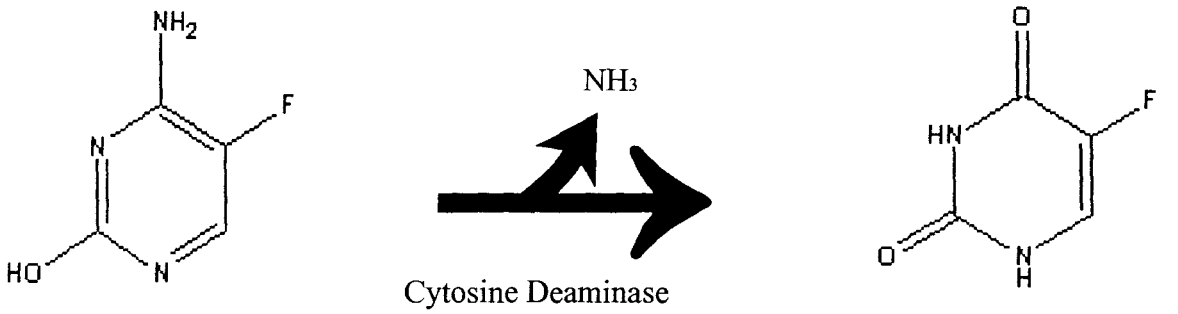
Based on its selectivity, a primary application of suicide gene therapy is the treatment of cancer. This reflects the objective of cancer treatment, which is to eliminate malignantly transformed cells while inflicting minimal damage elsewhere within the patient. However, use of suicide gene therapy in cancer has been limited mostly to solid tumours because of the limitation set by the need for a distinct target into which the vector can be delivered. Some suicide gene therapy approaches for haematologic malignancies are under development (Dilber & Gahrton, 2001).

Of all the suicide gene therapy strategies being tested for the treatment of cancer, two have been the object of more focused developmental effort and are currently in clinical trials (Morris *et al.*, 1999). First, is the combination of herpes simplex virus thymidine kinase (HSV-tk) and ganciclovir or acyclovir. The enzyme tk converts the antiviral nucleoside agents ganciclovir (gcv) and acyclovir (acv), to their respective monophosphate forms (Mullen, 1994). To date, considerable progress has been made in the development of the HSV/tk + acv/gcv combination resulting in 60 clinical trials on-going at the time of thesis publication (www.wiley.co.uk/genmed). The second suicide gene therapy is the combination of *E.coli* cytosine deaminase (CDA) plus 5-fluorocytosine (5FC). CDA deaminates the antimycotic agent 5FC to 5-fluorouracil (5FU) (Figure 1.1). CDA/5FC has been the focus of a more modest effort and as a result its clinical applications are not as far advanced. It is, however, the subject of 2 clinical trials on-going at the time of thesis publication (www.wiley.co.uk/genmed). Further description of suicide gene therapy strategies will be limited to the CDA/5FC system.

Cytosine Deaminase and 5-Fluorocytosine

The enzyme CDA is expressed in some bacteria and fungi, not in mammalian cells, and catalyzes the deamination of cytosine to uracil. CDA was first cloned and sequenced in 1989 (Andersen *et al.*, 1989, Kilstrup *et al.*, 1989). The first demonstration of its use as a suicide gene was in 1992 by Austin and Huber, for the treatment of colorectal cancer metastases (Austin & Huber, 1993). CDA is encoded by the CodA gene of *E. coli* and is activated under circumstances of nutritional stress (Andersen *et al.*, 1989). CDA expression is repressed in conditions of excess purines and derepressed in conditions of limiting nitrogen and pyrimidines (Kilstrup *et al.*, 1989). In suicide gene therapy, CDA from *E.coli* is used almost exclusively, although CDA from *Saccharomyces cerevisiae* has also been used in combination with 5FC (Kievit *et al.*, 1999).

Figure 1.1. *The deamination of non-toxic 5-fluorocytosine (5FC) to highly toxic 5-fluororuracil (5FU) by cytosine deaminase (CDA).*



5-Fluorocytosine

5-Fluorouracil

5FC was first synthesized by Heidelberger et al in 1957 (Vermes *et al.*, 1957). It is a synthetically produced, fluorinated pyrimidine composed of a cytosine molecule with an added fluorine moiety. It is a small non-polar molecule that diffuses through the cell membrane into the cytoplasm (Vermes *et al.*, 2000). Halogenation of cytosine confers cytotoxic potential on this otherwise biologically permissive molecule.

Originally, 5FC was considered for use as an anti-cancer agent but the effort was unsuccessful, however, its potential as an antifungal agent was soon revealed and since then the primary clinical application of 5FC has been as an agent in the treatment of fungal infections in mammals (Vermes *et al.*, 2000). Interestingly, with the invention of suicide gene therapy, 5FC is emerging once again as an agent for the treatment of cancer.

5-Fluorouracil

5FU is also a fluorinated pyrimidine analogue however, unlike 5FC, it is highly cytotoxic. For over 40 years 5FU has been used as an antineoplastic, most notably for the treatment of colorectal cancer but also for cancers of the breast and pancreas, among others (Myers *et al.*, 1976) (Grem, 2000). Currently, 5FU is administered directly to patients via any one of a number of different bolus and continuous infusion schedules. In the context of suicide gene therapy, however, 5FU is generated intracellularly as the product of the CDA mediated deamination of 5FC.

Prior to inflicting cytotoxic effects within the cell, 5FU must be metabolized further to 5-fluorodeoxyuridine monophosphate (FdUMP), 5-fluorouridine triphosphate (FUTP), and 5-fluorodeoxyuridine triphosphate (FdUTP) (Schmoll *et al.*, 1999) (Myers *et al.*, 1976). A complex network of enzymatic reactions is involved in the generation of these metabolites, each of which eventually targets a different biochemical process within the cell. These are, respectively, inhibition of thymidylate synthase (TS) (Diasio *et al.*, 1978), misincorporation into RNA (Wilkinson & Crumley, 1977), and misincorporation into DNA (Ingraham *et al.*, 1980) (Figure 1.2). The cytotoxicity of 5FU is related to the inhibition of TS by FdUMP and misincorporation of FUTP into RNA, but not to misincorporation of FdUTP into DNA (discussed below). Misincorporation into DNA is thought to

occur with such extreme rarity that it is not relevant to the overall cytotoxic effect of 5FU (Ingraham *et al.*, 1980).

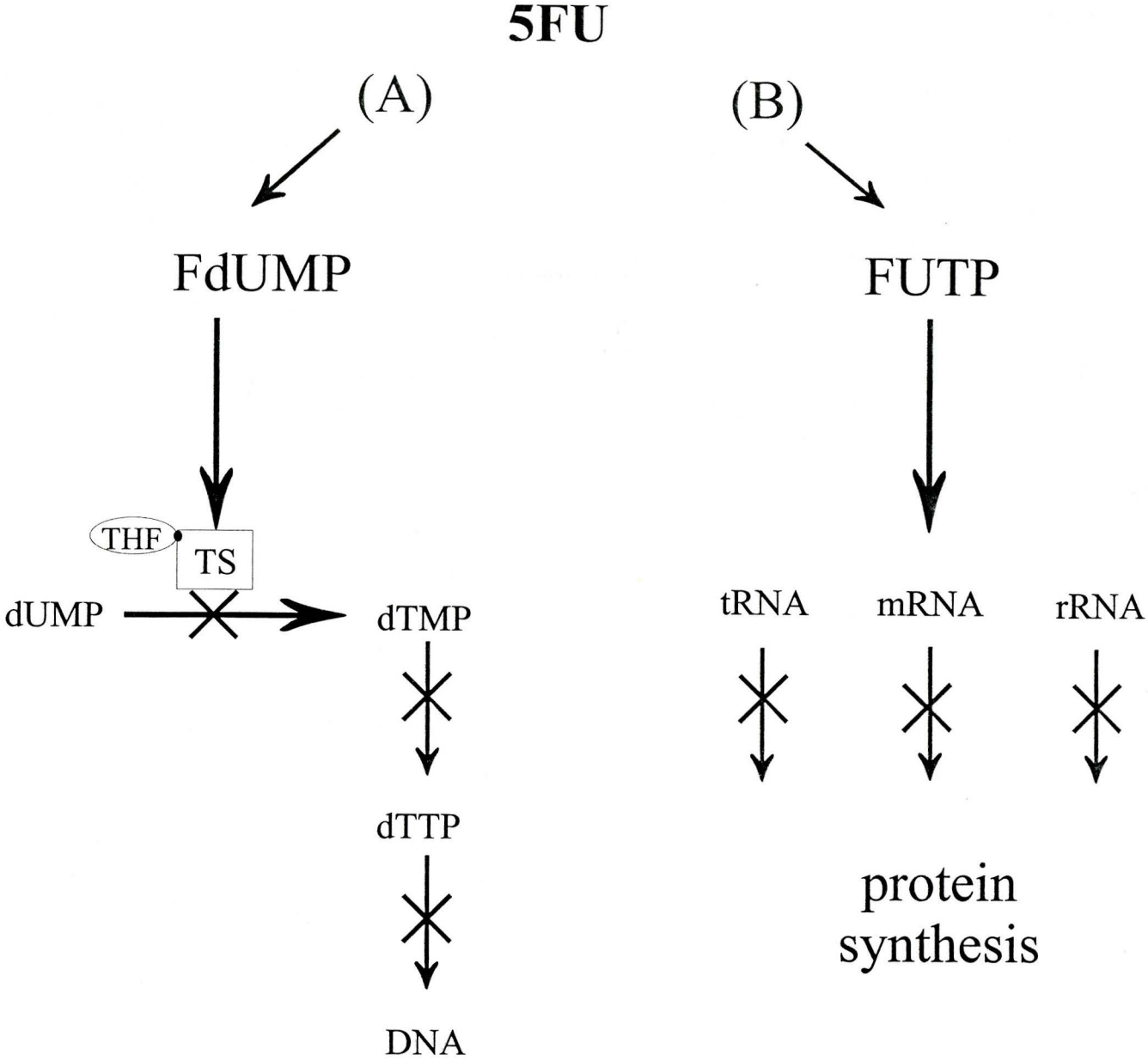
TS catalyzes the *de novo* synthesis of deoxy-thymidine monophosphate (dTMP) from deoxy-uridine monophosphate (dUMP) in the presence of the co-factor 5, 10-methyl tetrahydrofolate (THF) (Anderson *et al.*, 1999). This is a two-step reaction in which a CH_2 group is transferred from THF to dUMP and then reduced to CH_3 (Danenberg, 1977). In the absence of THF, FdUMP acts as a reversible inhibitor for the binding of dUMP to TS; however, when THF is present, FdUMP forms a covalent bond with TS (Danenberg, 1977). The resulting tertiary structure sequesters TS, rendering it unavailable to catalyze the conversion of dUMP to dTMP. The absence of dTMP is lethal because the cell cannot make deoxy-thymidine triphosphate (dTTP), thus preventing DNA synthesis, repair and replication.

The extent of TS inhibition is directly related to levels of FdUMP and THF within the cell. Generation of FdUMP depends on the amount of 5FU taken up by the cell as well as the intracellular levels of those enzymes involved in its conversion to FdUMP. These events occur in a cell specific manner (Weckbecker, 1991). Cells with

Figure 1.2. *Intracellular metabolism of 5-fluorouracil (5FU) to 5'-fluorodeoxyuridine monophosphate (FdUMP) and 5'-fluorouridine triphosphate (FUTP). Once within the cell, 5FU is metabolized to the cytotoxic compounds FdUMP and FUTP.*

A. FdUMP binds to the enzyme thymidylate synthase (TS). In the presence of the co-factor 5'10'-methyltetrahydrofolate (THF) a covalent bond forms between FdUMP and TS. This tightly bound complex effectively sequesters the enzyme, inhibiting its catalysis of deoxythymidine monophosphate (dUMP) formation. Without dUMP the cell cannot make *de novo* deoxythymidine triphosphate (dTTP) which is necessary for DNA synthesis.

B. FUTP misincorporation into transfer RNA (tRNA) interferes with amino acid chain elongation. Misincorporation into messenger RNA (mRNA) inhibits or enhances transcription, interferes with mRNA splicing, and causes mispairing of bases during mRNA translation. Misincorporation into ribosomal RNA (rRNA) negatively affects protein synthesis.


5FU
(A)
(B)
FdUMP
FUTP
THF
TS
dUMP
dTMP
dTTP
DNA
tRNA
mRNA
rRNA
protein
synthesis

increased levels of THF are more sensitive to the FdUMP-directed cytotoxic mechanisms of 5FU because there is an increased formation and less dissociation of the ternary complex (Danenberg & Lockshin, 1982). Cells with decreased levels of dUMP are also more sensitive to FdUMP directed action. Since dUMP and FdUMP compete for binding to TS, the lower the intracellular concentration of dUMP, more likely it is that FdUMP will bind to TS (Myers *et al.*, 1975). Finally, cells with low concentrations of TS are also more sensitive to FdUMP. Comparison of TS levels in HT29 and HuTu80 human colon carcinoma cell lines revealed that lower TS levels in the HT29 cells were associated with the sensitivity of these cells to 5FU, while the relatively resistant HuTu80 cells had markedly increased TS levels (Washtien, 1984).

The second cytotoxic metabolite of 5FU is FUTP. Cytotoxicity of FUTP is related to its incorporation into RNA. Incorporation of FUTP into messenger RNA (mRNA) has been shown to inhibit or stimulate transcription (Glazer & Peale, 1979), to interfere with mRNA splicing (Heidelberger *et al.*, 1983), to cause mispairing of bases during translation of mRNA (Dolnick & Pink, 1985) and to interfere with post-translational modifications (Jin *et al.*, 1996). FUTP can also be incorporated into ribosomal RNA (rRNA) (Wilkinson & Pitot, 1973) or transfer RNA (tRNA) (Weckbecker, 1991) which negatively affect protein synthesis and amino acid chain elongation, respectively. The inhibition of mRNA is thought to be the primary RNA-directed effect of FUTP (Pinedo & Peters, 1988).

Regarding the DNA-directed effects of 5FU, despite the existence of biochemical pathways to generate FdUTP, it is extremely difficult to detect, in the cytoplasm or in DNA. It may not be detectable at all. Its removal from DNA is mediated efficiently by deoxyuridine triphosphate diphosphohydrolase and uracil-DNA glycosylase, which act to hydrolyze FdUTP and excise it from DNA, respectively (Ingraham *et al.*, 1980).

Cytosine Deaminase plus 5-Fluorocytosine Suicide Gene Therapy

Sensitization of mammalian cells to 5FC with CDA was first demonstrated by Mullen *et al*, in 1992. Here, the CDA gene underwent PCR oligonucleotide directed mutagenesis to adjust the transcriptional start site and enhance its expression in the eukaryotic system. Transfer of the modified CDA gene into untransformed murine fibroblast cells conferred a lethal sensitivity to 5FC that was not

observed in untransduced cells exposed to 5FC. Since this seminal report, CDA/5FC suicide gene therapy has been applied to a wide variety of murine and human cells. Although this therapy has achieved differential success in its application to various cell lines, a significant, informative body of research has emerged.

Sensitization to 5FC by CDA can be achieved in a variety of murine, rat, and human cancer cell types including: murine colorectal carcinoma (Mullen *et al.*, 1994) (Kuriyama *et al.*, 1999) (Block *et al.*, 2000), hepatocellular carcinoma (Kuriyama *et al.*, 1998) (Kuriyama *et al.*, 1999) and mammary carcinoma (Consalvo *et al.*, 1995); rat gliosarcoma (Dong *et al.*, 1996), squamous cell carcinoma (Kuriyama *et al.*, 1998) and colorectal carcinoma (Pierrefite-Carle *et al.*, 1999); human breast cancer (Li *et al.*, 1997), colorectal carcinoma (Huber *et al.*, 1994) (Hirschowitz *et al.*, 1995) (Ohwada *et al.*, 1996) (Rowley *et al.*, 1996) (Block *et al.*, 2000) (Koyama *et al.*, 2000), gliobastoma (Rowley *et al.*, 1996), and renal cell carcinoma (Shirakawa *et al.*, 1999).

In addition to the sensitization to 5FC conferred by CDA suicide gene therapy some additional characteristics of this therapy have been described. First of all, there is a significant bystander effect associated with CDA/5FC therapy *in vitro* (Kuriyama *et al.*, 1998) and *in vivo* (Huber *et al.*, 1994). The term 'bystander effect' refers to the killing of tumour cells that have not been transduced by the suicide gene. This is facilitated by the diffusion of 5FU from the cell in which is was converted by CDA into nearby cells that do not express the enzyme and thus cannot generate 5FU intracellularly. Of note, the bystander effect is not unique to CDA/5FC and has been observed in HSV/tk suicide gene therapy (Dilber & Smith, 1997). The difference between the bystander effect in these two systems is that, while 5FU can freely diffuse from cell to cell, the transport of the highly-charged GCV or ACV requires the formation of gap junctions between adjacent cells. It is thought that this requirement is a limiting factor in the success of tk therapy.

The bystander effect has been demonstrated *in vitro* in several ways. In cell mixing experiments the proportion of CDA-transduced to untransduced cells is variable. These experiments demonstrate that it is not necessary for 100% of cells to express CDA to achieve significant reductions in cell survival (Rowley *et al.*, 1996). Alternatively, 5FU is detectable in media conditioned by CDA-expressing cells cultured in the presence of 5FC. When it is transferred to untransduced cells their

survival is reduced in proportion to the amount of 5FU detected in the conditioned media (Kuriyama *et al.*, 1998). Similar to *in vitro* cell mixing experiments, mixed populations of transduced and untransduced cells have been used to form tumours in animals. It has been shown that even when tumours contained only 30% transduced cells, regression was achieved that was similar to the effect observed if 100% of cells expressed CDA (Huber *et al.*, 1994). *In vivo,* the bystander effect is more difficult to delineate as clearly as it is *in vitro*. The identification of an immune component to CDA/5FC therapy, described below, confuses the definition of 'bystander'. Determining which proportion of the total anti-tumour effect of the therapy attributable strictly to the biochemical cytotoxicity of 5FU compared to that which is attributable to immune-directed mechanisms is difficult. Nonetheless, *in vitro* data intimate the existence of bystander effect of CDA/5FC, *in vivo*.

Several studies have demonstrated the involvement of immune mechanisms in CDA/5FC suicide gene therapy based on the use of retroviral vectors expressing CDA. Part of the immune response is thought to be related to the induction of apoptosis by 5FU (Consalvo *et al.*, 1995). This generates cellular debris that can be processed and presented by antigen presenting cells such as dendritic cells. It has been shown repeatedly that tumour-bearing animals treated with CDA/5FC therapy are protected against tumour growth following tumour cell re-challenge (Consalvo *et al.*, 1995) (Mullen *et al.*, 1994). Histological analysis of CDA/5FC treated tumours revealed infiltration of CDA/5FC treated tumours by lymphocytes, specifically CD8+ T cells, granulocytes (Kuriyama *et al.*, 1999), and natural killer cells (Pierrefite-Carle *et al.*, 1999). Furthermore, it has been shown that antibody mediated depletion of CD8+ T cells abrogated the anti-tumour effect of the CDA/5FC therapy. Finally, Kuriyama et al, 1999 demonstrated that while CDA/5FC therapy was effective against tumour growth in immune-competent mice, it did not affect tumour growth in athymic nude mice. In addition, the immunogenicity of CDA, a protein not normally present in mammalian cells, has also been linked to the immunostimulatory properties of CDA/5FC therapy. In fact, several groups have demonstrated that concurrent expression of CDA and either interleukin-6 (Il-6) (Mullen *et al.*, 1996) or interferon-γ (IFN-γ) (Nanni *et al.*, 1998) is an effective anti-cancer treatment with or without the addition of the prodrug 5FC.

Cisplatin

Cisplatin (*cis*-dichlorodiammineplatinum II) is a square planar molecule composed of a central platinum (II) ion surrounded by two ammonia groups and two chloride atoms, positioned in the cis-arrangement (Rosenberg, 1985) (Figure 1.3). Cisplatin was first described for its ability to inhibit cell division in *E.coli,* but it was not until several years later that its properties as an anti-cancer agent were identified (Rosenberg, 1985). Cisplatin is now used routinely in the treatment of cancer, most notably for ovarian, cervical, bladder, testicular, small and non-small cell lung, and head & neck cancers (Kirkwood *et al.*, 1998). Cisplatin is considered a non-classical alkylating agent since, although it induces crosslinks in DNA, it does so in a manner that is different from the classical alkylating agents (Kirkwood *et al,* 1996).

Cisplatin is administered intravenously to patients and, in plasma, it exists as a stable, non-reactive compound. It is thought that cisplatin enters cells by passive diffusion, however there is evidence to support the existence of protein-mediated transport mechanisms (Gately & Howell, 1993). Once within the cell, where the chloride concentration is approximately 30 times lower than it is in plasma, cisplatin undergoes a hydration reaction in which both chloride ions are replaced by water molecules (Chu, 1994). This transforms the molecule into a highly reactive, positively charged electrophile which proceeds to react with DNA and protein (Kirkwood *et al.,* 1996). These interactions result in interstrand and intrastrand complexes between platinum ions and DNA, and DNA and protein (Figure 1.4) which cause severe distortion and unwinding of the DNA double helix (Takahara *et al.*, 1995). Cytotoxicity of cisplatin is thought to be primarily related to the formation of 1,2-d(GpG) and 1,2-d(ApG) intrastrand crosslinks, which account for 65% and 25%, respectively, of all cisplatin-DNA lesions in cisplatin treated cells (Chu, 1994).

Damage to DNA leading to the interference and/or inhibition of DNA synthesis and transcription are the mechanisms by which cisplatin exerts its cytotoxic effects (Zlatanova *et al.*, 1998). While the effects of cisplatin are cell-cycle nonspecific (Kirkwood *et al.*, 1998), the resulting DNA damage is thought to cause arrest in G2 of the cell cycle (Chu, 1994). In order to progress in the cell cycle to M-phase, the cell must repair this damage. Nucleotide excision repair is the DNA repair pathway considered responsible for repair cisplatin-DNA crosslinks, since 90% of these lesions are intrastrand,

rather than interstrand, crosslinks (Chu, 1994, Zamble *et al.*, 1996). Excision repair involves the identification of cisplatin-DNA crosslinks, followed by the removal of that region of DNA and its subsequent resynthesis (Zlatanova *et al.*, 1998, Lodish *et al.*, 1995). If cells are unable to repair the cisplatin-DNA adducts cell cycle progression is halted and cell death results through the initiation of necrotic and apoptotic pathways (Gonzalez *et al.*, 2001).

Figure 1.3. *Cis-dichlorodiammineplatinum II (cisplatin)*
Cisplatin is a square planar molecule composed of a central platinum (II) ion surrounded by two ammonia groups and two chloride atoms, positioned in the cis-arrangement.

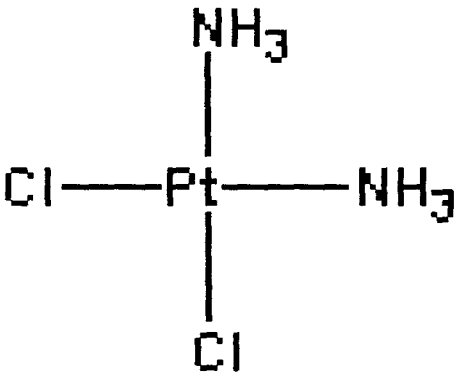
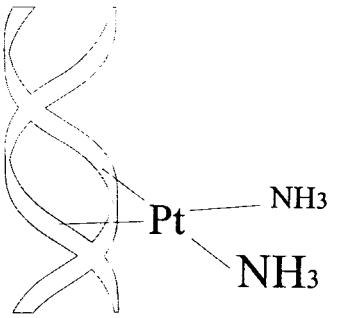
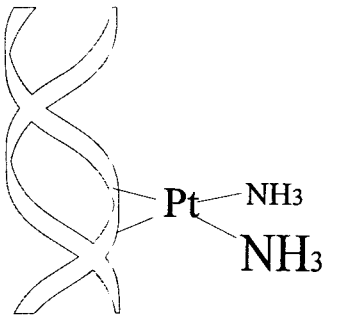

NH3
Cl
Pt
NH3
Cl

Figure 1.4. *Interactions of cisplatin with DNA and protein.*
A. Interstrand crosslink
B. Intrastrand crosslink
C. DNA-protein crosslink



A.



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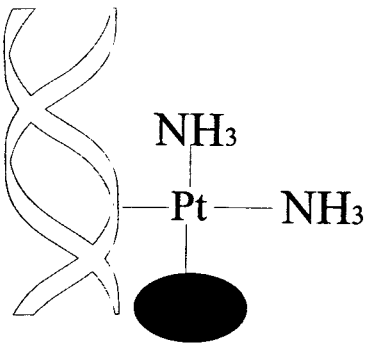


C.

Despite its successful application to the treatment of a variety of tumours, resistance to cisplatin remains a significant limitation to its curative potential. Resistance can be innate, as is the case for cancers of the colon and pancreas, or it can be acquired following initial courses of treatment to cisplatin-sensitive tumours (Perez, 1998). A diversity of mechanisms have been proposed to explain cisplatin resistance observed both *in vitro* and *in vivo*. These include mechanisms by which cells decrease uptake or increase efflux of cisplatin (Chu, 1994), or increase the repair of cisplatin damaged DNA (Perez, 1998). Significant steps have been taken toward understanding the molecular and genetic determinants of cisplatin resistance. While pre-clinical studies continue to elucidate mechanisms of cisplatin resistance, the current solution to this problem, clinically, has been to adjust treatment regimens to accommodate the likelihood of resistance by combining cisplatin with other chemotherapy drugs or with radiotherapy.

Combination Cancer Therapy: 5-Fluorouracil & Cisplatin

With few exceptions, the use of treatment regimens that incorporate multiple antineoplastic agents is necessary in the treatment of cancer (Frei *et al.*, 1998). The requirement for incorporating different anticancer agents into a single treatment regimen is based on the genetic heterogeneity and instability of malignantly transformed cells. In any given tumour, a proportion of cancer cells may be inherently resistant to a single treatment and others become refractory during treatment, ultimately leading to treatment failure (Schnipper, 1986). As the biochemical mechanisms of cytotoxicity for different antineoplastics are better elucidated, combinations can be devised rationally, based on non-overlapping mechanisms, rather than empirically, according to observations from clinical practice (Peters *et al.*, 2000a). The use of several agents to treat a single cancer is validated because it provides a more aggressive attack on the tumour compared to use of a single agent. The development of resistance to more than one agent, when they are administered concurrently, is less likely to occur than the development of resistance to a single agent.

The combination of 5FU and cisplatin was developed in response to the severe dose-limiting cytotoxicity of 5FU. This combination has been tested clinically according to a variety of dosing and administration schedules for the treatment of several different cancers. Specifically it has been used for

the treatment of patients with squamous cell carcinomas of the esophagus and head & neck (Amrein & Weitzman, 1985) (Decker *et al.*, 1983) (Rooney *et al.*, 1985), small cell cancer of the lung (Morere *et al.*, 1994), colon (Loehrer *et al.*, 1985) and gastric cancer (Lacave *et al.*, 1991). This combination is not ubiquitously beneficial. Several studies have shown that it did not confer any benefit compared to existing treatment regimens in the treatment of adenocarcinoma of the stomach (Williamson *et al.*, 1995), colon (Kemeny *et al.*, 1990) and esophagus (Levard *et al.*, 1998). Documented clinical experience is inconclusive as to whether this combination is effective, and if so, what would be an optimal protocol for administration. In response, pre-clinical research has attempted to elucidate the observed synergistic interaction of these two agents that appears to be both tumor cell- and regimen-specific. There is on-going debate regarding these issues (Johnston *et al.*, 1996, Scanlon *et al.*, 1986).

There are two basic theories for this observed synergism, each of which refers to a particular sequence of drug administration. The sequence of cisplatin followed by 5FU has been shown to act synergistically in human head & neck and ovarian cancer cell lines (Shirasaka *et al.*, 1993) (Scanlon *et al.*, 1986). This is explained by the fact that cisplatin inhibits the uptake of methionine into the cell. This causes a perturbation of cellular methionine pools and subsequently upregulates the production of folates within the cell. Production of the folate co-factor 5, 10-methyltetrahydrofolate (THF), which facilitates the covalent interaction between TS and its inhibitor FdUMP, is also upregulated. When the cell is treated with 5FU the increased level of THF causes increased formation of the TS:FdUMP:THF ternary complex, potentiating the inhibition of thymidine production within the cell, which leads to greater cytotoxicity of the treatment. This particular sequence of administration seems to depend primarily on the TS cycle within the target cell.

Alternatively, it has been shown that the sequence of 5FU followed by cisplatin is also synergistic (Johnston *et al.*, 1996) (Esaki *et al.*, 1992) (Palmeri *et al.*, 1989). In this system pre-exposure to 5FU causes both inhibition of TS and incorporation of FUTP into RNA. These events ultimately interfere with the repair of cisplatin-induced DNA damage. Inhibition of TS causes a reduction in the thymidine available for repair of cisplatin-damaged DNA (Johnston *et al.*, 1996). The incorporation of FUTP into RNA interferes with the nuclear localization and transport of mRNA, specifically for enzymes, such as ERCC1, required for DNA repair of cisplatin damage (Esaki *et al.*, 1992).

Combining AdCDA/5FC & Cisplatin

Reports of 5FU/cisplatin combination therapy provide an important precedent upon which to evaluate the effect of combining AdCDA/5FC and cisplatin. This combination acknowledges not only the necessity for cancer treatments to incorporate more than one agent, but also the undesirable toxicity and morbidity to the patient associated with systemic administration of these agents. Using AdCDA/5FC as a substitute brings with it several unique, gene therapy-specific advantages. Systemic administration of 5FC and direct, intra-tumoural injection of the AdCDA vector facilitates generation of 5FU in the vicinity of the tumour, with little exposure to tissues elsewhere in the body. This is advantageous in comparison to the effects of systemic 5FU administration. As well, there is the added component of immune stimulation resulting from the use of an Ad vector to deliver a non-mammalian metabolic enzyme, effectively an immunogenic non-self protein, to human cells. While there exists no precedent in the literature for this particular combination of treatments, several other combination therapies support the one proposed here. There is, for example, the observation of the synergistic interaction between 5FU and cisplatin, in addition to the combination of AdCDA/5FC with radiation therapy (Khil *et al.*, 1996) (Szary *et al.*, 1997) (Hamstra *et al.*, 1999) and with cytokine gene therapy, described above. This study evaluates the combination of AdCDA/5FC suicide gene therapy and cisplatin chemotherapy, *in vitro*.

CHAPTER 2

MATERIALS & METHODS

Cell Lines

The MT1A2 cell line is a murine mammary adenocarcinoma tumour cell line established from tumours arising in the mammary epithelium of transgenic Polyoma middle T (PyMT) mice. These mice express the PyMT oncogene under control of the mouse mammary tumour virus (MMTV) LTR (Guy *et al.*, 1992).

The B16/F10 cell line is a chemically transformed, highly metastatic murine melanoma cell line from mice of the C57Bl/6J background (Fidler & Kripke, 1977). They were kindly provided by Dr Y. Wan, McMaster University.

HT29 cells are human colorectal adenocarcinoma cells (Fogh *et al.*, 1977). HT29p14 cells are an artificially derived sub-line of HT29 cells. They have been made resistant to the photodynamic therapy sensitizing agent photofrin following 14 passages in this agent (Singh *et al.*, 2001). Photofrin is the chemotherapeutic agent used in photodynamic therapy (PDT) (Dougherty *et al.*, 1998). Both HT29 and HT29p14 cells were kindly provided by Dr G. Singh, Hamilton Regional Cancer Centre, Hamilton, Ont.

Recombinant Adenovirus Constructs

All adenovirus (Ad) constructs included in this work are first generation, serotype-5 Ad (Ad5) vectors. First generation vectors have deletions of the E1 and E3 regions (Hitt *et al.*, 1997). AdCDA contains the *E.coli* CDA gene in the deleted E1 region under control of the murine cytomegalovirus (MCMV) immediate early promoter and followed at the 3' end by the Simian virus 40 polyadenylation sequence (SV40pA). AdLacZ contains the *E.coli* β-galactosidase gene in the deleted E1 region under control of the MCMV promoter (Mittal *et al.*, 1993). Ad-dl70-3 is a first generation adenovirus that does not contain a transgene in the E1 region (Bett *et al.*, 1994). Recombinant vectors were grown up

and cesium chloride gradient purified by members of the laboratories of Dr. Frank Graham and Dr. Jack Gauldie, as described in Hitt *et al* (1995).

Reagents

5-fluorocytosine (5-FC) was obtained from Sigma (St. Louis, Missouri, USA; cat # F7129; lot # 19H4072) and from ICN Pharmaceuticals (Montreal, Quebec; lot # SI 98110042). Cisplatin was obtained from Faulding (Vaudreuil, Quebec; DIN # 02126613), and 5-fluorouracil (*Adrucil*) from Pharmacia & Upjohn (Mississauga, Ontario; DIN # 02063921). Trypan blue was obtained from GibcoBRL (cat # 15250-061). Methylene blue was obtained from Sigma (St. Louis, Missouri, USA; cat # M4159).

Methods

Cell Culture

All cell culture media was obtained from GibcoBRL and prepared in the Department of Pathology central facility.

All cell cultures were maintained at 37°C with 5% CO_2. MT1A2 cells were cultured in Dulbecco's medium (DUL; cat # 12800-082) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, Missouri; cat # F-4135), 100 g/ml penicillin and 100U/ml streptomycin (Pen/Strep; GIBCO BRL; cat # 15140-122), 2 mM L-glutamine (L-glu; Sigma, St. Louis, Missouri; cat # G-8540), and 30ng/ml epidermal growth factor (EGF; GIBCO BRL; cat # 53003-018). B16/F10 cells were cultured in minimum essential medium F-11 (MEM F11; cat # 61100-087), 10% FBS, 100 g/ml penicillin, 100U/ml streptomycin, 2 mM L-glu, 1.5% MEM Vitamin Solution (GibcoBRL; cat # 11120-052), 1 mM MEM Non-Essential Amino Acids (GibcoBRL; cat # 11140-050), 10 mM sodium pyruvate solution, 10 mM HEPES buffer solution. HT29 and HT29p14 cells were cultured in α-minimal essential medium (α-MEM; GIBCO BRL; cat # 1200-063) supplemented with 10% FBS, 100 g/ml penicillin, 100U/ml streptomycin, and 2 mM L-glu.

Cells were passaged at 2 to 4 day intervals by rinsing the dish with phosphate buffered saline (PBS) and applying 2x Trypsin-EDTA (GIBCO BRL; cat # 15400-054) for 2 minutes at 37°C. Cells were split at ratios between 1:2 and 1:5.

Preparing Cells for Storage in Liquid Nitrogen

Cells were trypsinized for 3 minutes at 37°C with 5% CO_2, suspended in 20 ml media/150 mm culture dish, and centrifuged at 1500 rpm for 5 minutes. The supernatant was aspirated and the pellet was resuspended in 1 ml FBS plus 10% dimethyl sulfoxide (DMSO; Sigma, St. Louis, Missouri, USA;cat # D2650) per 150 mm dish. 1ml of the cell suspension was aliquoted into Nalgene Cryotubes (Rochester, New York, USA; cat # 5000-0020), placed into an isopropanol-filled, Nalgene Cryo 1°C Freezing Container (Rochester, New York, USA; cat # 5100-0001) and transferred directly to –70°C. Cells were transferred to liquid nitrogen at a later date.

Standard Protocol for Adenovirus Infection

Twelve to 16 hours prior to infection, cells were trypsinized and counted by trypan blue exclusion. Cells were seeded into 24-well plates (Becton Dickinson, Franklin Lakes, New Jersey, USA; cat # 353047) to achieve 80%-90% confluence by the following morning. MT1A2 cells were seeded at 10^5/well, F10 cells at 1.5 x 10^5/well, HT29 cells at 4 x 10^5/well and HT29p14 cells at 1.5 x 10^5/well. Immediately prior to infection a representative well was trypsinized and counted. This value was assumed to represent other wells seeded at the same time, and was used to calculate the volume of virus needed to achieve the desired multiplicity of infection (MOI). Monolayers were aspirated, overlaid with 0.2 ml virus dilution in PBS ++, then incubated for 60 minutes at 37°C and 5% CO_2. After this time the virus was aspirated and 1 ml of fresh media was replaced in each well.

Preparation of 5FC

5FC was prepared 30 minutes prior to commencing the clonogenic assay. It was prepared at 2x the desired concentration and at half the volume required for the experimental protocol, to be diluted by half upon addition of the cell suspension.

An appropriate mass of 5-FC was weighed directly into a 100 ml bottle. The correct volume of media, and a magnetic stir bar were added. The bottle was placed in a 37°C water bath for 15 – 25

minutes, then placed on a stirring-plate for 10 minutes. The 5FC was filter sterilized (0.22uM, Nalgene, Rochester, New York, USA; cat # 121-0020) prior to use in cell culture.

Clonogenic Assay: AdCDA/5FC Treatment

The clonogenic assay worksheet (Appendix I) details the procedures and calculations included in a clonogenic assay for evaluating either the AdCDA/5-FC single treatment or the AdCDA/5-FC + cisplatin combination treatment regimen. All single- and combination-treatment categories were repeated in triplicate (3 wells) in each experiment.

3 hours following the end of the infection period cells were trypsinized for 2 minutes with 0.2 ml of 1x trypsin and 1.8 ml of media then was added to each well. Each well was counted and an appropriate volume of the cell suspension was diluted in an appropriate volume of media. The cell suspensions were prepared as follows: MT1A2 cells at 50 cells/ml, F10 cells at 50 cells/mL, HT29 cells at 200 cells/ml, and HT29p14 cells at 50 cells/ml.

Infected cells were then seeded into 6 well plates (Becton Dickinson; Franklin Lakes, New Jersey, USA; cat # 353406) at 100 cells/well for MT1A2, F10 and HT29p14 cells and 400 cells/ml for HT29 cells together with 4 ml of growth medium containing an appropriate concentration of 5FC. This constitutes the beginning of day 0.

On day 2 of the experiment media was aspirated from all wells and replaced with fresh media, not containing 5FC. Cells were allowed to culture for a further 3 (F10 cells) or 5 (MT1A2, HT29, HT29p14 cells) days at which time colonies were stained with methylene blue and counted at 40x magnification using a dissecting microscope.

Clonogenic Assay: Cisplatin Treatment

Confluent cells were trypsinized and counted by trypan blue exclusion. Cells were then seeded into 6 well plates at 200 cells/well for MT1A2, HT29 and HT29p14 cells and 100 cells/well for F10 cells together with 2 ml of growth medium. At 3 h after seeding the growth medium was aspirated and fresh growth media containing an appropriate concentration of cisplatin was added

Cisplatin was obtained from a fresh vial for each experiment and solutions were prepared in growth media immediately prior to use. Following treatment of cells with cisplatin for 1 h at 37°C with 5% CO_2, the media was aspirated, the cells were rinsed with 6 ml of PBS and 4 ml of fresh growth media without cisplatin was added. Cells were then incubated for a further 5 days (F10 cells) or 7 days (MT1A2, HT29, and HT29p14 cells) at which time the cells were fixed, stained with methylene blue and counted at 40x magnification using a dissecting microscope.

Clonogenic Assay: 5FU Treatment

Confluent cells were trypsinized and counted by trypan blue exclusion. 2 ml per well of each cell suspension was seeded 6-well tissue culture plates and placed at 37°C with 5% CO_2 for 3 hours to cells to adhere. F10, MT1A2, HT29, and HT29p14 cells were seeded at 200 cells/ml.

5FU was obtained from a fresh vial for each experiment and solutions were prepared in growth media immediately prior to use. Following treatment of cells with 5FU for 48 h at 37°C with 5% CO_2, the media was aspirated, the cells were rinsed with 6 ml of PBS and 4 ml of fresh growth media without cisplatin was added. Cells were then incubated for a further 4 days (F10) or 5 days (MT1A2, HT29, and HT29p14 cells) at which time the cells were fixed, stained with methylene blue and counted at 40x magnification using a dissecting microscope

Clonogenic Assay: Combination AdCDA/5FC + Cisplatin Treatment

The protocol for AdCDA/5FC treatment was followed exactly as described above. On day 2, coincident with the aspiration of media containing 5FC media, cells were treated with a range of concentrations of cisplatin in 2 ml fresh media as described above. After which time cells were returned to culture for 5 days (F10 cells) or 7 days (MT1A2, HT29, and HT29p14 cells). Colonies were stained with methylene blue and counted at 40x magnification using a dissecting microscope.

β-Galactosidase Assay

Cells at 75% confluence were infected at moi 0, 10, 50, 100 with AdCA35LacZ virus according to the standard infection protocol. Infected cells were then cultured for 3 days at 37°C with 5% CO_2. At

the end of the infection period, for each well, media was aspirated and 1 ml PBS + 3 mM EDTA was applied for 5 minutes at 37°C with 5% CO_2. Cells were then scraped gently and transferred to 1 ml Eppendorf tubes and centrifuged for 10 minutes at 1500 x g. Pellets were resuspended in media and centrifuged for 5 minutes at 4000 x g. Pellets were aspirated in 100 µl freshly prepared X-gal reagent (25µl 2% X-gal; 67µl 30 mM K3F5(CN)6; 67µl 30 mM K45(CN)6; 1µl 1 M MgCl2; 341µl PBS). X-gal cell suspensions were incubated for 2 h at 37°C with 5% CO_2, and stored for 2 – 24 h at 4°C. Both total cells and blue-staining cells were enumerated using a haemocytometer.

Intracellular Conversion of 5FC to 5FU

12 – 16 hours prior to infection cells were seeded in 12-well plates (Becton Dickinson; Franklin Lakes, New Jersey, USA; cat # 353043) at a density of 1.5x that used to seed cells in 24 well plates. Adenovirus infection was carried out according to the standard protocol. F10 cells were infected at an MOI of 50 and MT1A2 cells were infected at an MOI of 5. 3 hours following the end of the infection, media was replaced with 2 ml media containing 0 mM or 5 mM 5FC, prepared at the exact volume and concentration needed. Immediately after aliquoting 5FC, 50 µl media was removed from each well and added to 1 ml 1N HCl. This was repeated on days 1, 2, and 3. Samples stored at –20°C until all samples were collected.

Using UVette disposable cuvettes (Eppendorf Scientific, Westbury, New York, USA; cat # 952-01-005-1), the optical density of each sample was read at 255 nm and 290 nm against a blank of 50 µl fresh media in 1 ml 1N HCl. If OD readings exceeded 1.0, both the blank and the samples were diluted 10x in 1N HCl and re-read.

The concentrations of 5FC and 5FU were calculated according to the following equations (Wallace *et al.*, 1994):

$$5FC\ [mM] = 0.119\ (A_{290}) - 0.025(A_{255})$$

$$5FU\ [mM] = 0.185\ (A_{255}) - 0.049(A_{290})$$

Values obtained from these equations were multiplied appropriately to accommodate for dilution of the sample in HCl.

Statistical Analyses

Evaluation of the AdCDA/5FC + cisplatin combination treatment was evaluated as described in Caney *et al*, (2000). All graphed data represents the mean of three independent experiments and the corresponding standard error of the mean. A Student *t*-test compared survival of cisplatin treated AdCDA infected cells pre-exposed to 5FC or drug-free media. This test included values for surviving fraction that corresponded only to the higher of the two doses of cisplatin (16, 64, and 32 μM for MT1A2, HT29, and HT29p14 cells, respectively). The χ^2-goodness of fit test ($\Sigma(\chi^2)$) established the significance of the difference between the curves for combination and cisplatin-alone treatment groups (Bevington, 1969). This analysis included values for surviving fraction obtained for both doses of cisplatin used in each combination experiment.

CHAPTER 3

COMBINATION ADCDA/5FC SUICIDE GENE THERAPY OF MT1A2, HT29, AND HT29P14 CELLS

ABSTRACT

Gene therapy is emerging as a promising addition to the existing repertoire of anti-cancer treatments and its application as a single agent, or in combination with other agents is proving successful in both pre-clinical and clinical studies. Suicide gene therapy is a particular type of gene therapy used for the treatment of cancer. It involves the intracellular conversion of non-toxic prodrug to its active form by a metabolic enzyme of non-mammalian origin. There are many enzyme/prodrug combinations, one of which involves the bacterial enzyme cytosine deaminase (CDA) which converts inert 5-fluorocytosine (5FC) to highly toxic 5-fluorouracil (5FU). This work examines the combination of conventional cisplatin chemotherapy with cytosine deaminase + 5-fluorocytosine suicide gene therapy based on success achieved in the combination of 5FU and cisplatin. Using a first generation adenovirus type 5 vector expressing the *E. coli* cytosine deaminase (AdCDA) this combination was evaluated in murine mammary carcinoma MT1A2 cells, human colorectal carcinoma HT29 cells, HT29p14 cells, the photodynamic therapy (PDT) resistant sub-line of HT29 cells. Using a clonogenic assay, AdCDA infected cells were exposed to 5FC for 48 h and then to cisplatin for 1 h. Colonies were later stained and scored for viability. The effect of the combination on clonogenic survival was greater than additive compared to the sum of the two treatments administered separately. Evaluation of this treatment *in vivo*, using both murine and human tumor cell lines, will further define the potential of AdCDA/5FC + cisplatin as a clinically relevant cancer treatment.

Introduction

With few exceptions, the use of treatment regimens that incorporate different antineoplastic agents is absolute necessity in the treatment of cancer (Frei *et al.*, 1998). This requirement is based on the genetic heterogeneity and instability of malignantly transformed cells as these factors contribute to the development of drug-resistance. In any given tumour, a proportion of cancer cells may be inherently resistant to a single treatment and others become refractory during treatment, ultimately leading to treatment failure (Schnipper, 1986). As the biochemical mechanisms of cytotoxicity for different antineoplastics are better elucidated, combinations can be devised rationally, based on non-overlapping mechanisms rather than empirically, according to observations from clinical practice (Peters *et al.*, 2000a). Thus, the use of several agents to treat a single cancer is validated because it provides a more aggressive attack on the tumour compared to use of a single agent. Resistance to more than one agent when administered concurrently is less-likely to occur than the development of resistance to a single agent.

5-fluorouracil (5FU) and cisplatin are two well-established antineoplastic drugs used to treat a wide variety of cancers. Once within the cell, 5FU is metabolized by 5'-fluorodeoxyuridine monophosphate (FdUMP) and 5'-uridinetriphosphate (FUTP) (Schmoll *et al.*, 1999) (Myers *et al.*, 1976). The toxicity of 5FU is related to inhibition of thymidylate synthase (TS) by FdUMP, thus impairing *de novo* thymidine synthesis, and the misincorporation of FUTP into RNA which interferes with protein production (Diasio *et al.*, 1978, Wilkinson & Crumley, 1977). Cisplatin is a platinum based DNA damaging agent whose cytotoxicity is related the formation of intrastrand and interstrand crosslinks in DNA, as well as protein-DNA crosslinks (Takahara *et al.*, 1995). .

The combination of 5FU and cisplatin was developed in response to the severe dose-limiting cytotoxcity of 5FU. This combination has been tested clinically according to a variety of dosing and administration schedules for the treatment of several different cancers. Specifically it has been used for the treatment of patients with squamous cell carcinomas of the esophagus and head & neck (Amrein & Weitzman, 1985) (Decker *et al.*, 1983) (Rooney *et al.*, 1985), small cell cancer of the lung (Morere *et al.*, 1994), colon (Loehrer *et al.*, 1985) and gastric cancer (Lacave *et al.*, 1991). This combination is not

ubiquitously beneficial. Several studies have shown that it did not confer any benefit compared to existing treatment regimens in the treatment of adenocarcinoma of the stomach (Williamson *et al.*, 1995), colon (Kemeny *et al.*, 1990) and esophagus (Levard *et al.*, 1998). These reports of the clinical experience of combined 5FU and cisplatin do not permit conclusion as to the optimal protocol for administration or even whether this combination is beneficial. In response, pre-clinical research has attempted to elucidate the observed synergistic interaction of these two agents that appears to be both tumor cell and sequence specific (Johnston *et al.*, 1996) (Scanlon *et al.*, 1988). There is on-going debate regarding these issues.

As an extension of 5FU and cisplatin in combination, the current work seeks to evaluate the effect of combining AdCDA/5FC and cisplatin in murine and human cancer cells, in vitro. The proposed combination acknowledges not only the necessity for cancer treatments to incorporate more than one agent, but also the toxicity to the patient associated with the systemic administration of these agents. Using AdCDA/5FC as a substitute brings with it several unique, gene-therapy specific advantages. Systemic administration of 5FC and direct, intra-tumoural injection of the AdCDA vector facilitates generation of 5FU in the vicinity of the tumour, with little exposure to tissues elsewhere in the body. This is advantageous compared to the effects of systemic 5FU administration. As well, there is the added component of immune stimulation related to the use of an Ad vector to deliver a non-mammalian metabolic enzyme, effectively an immunogenic non-self protein, to human cells. While there exists no precedent in the literature for this particular combination of treatments, several other combination therapies support the one proposed here. First of all there is the observation of the synergistic interaction between 5FU and cisplatin. In addition, the combination of AdCDA/5FC with radiation therapy (Khil *et al.*, 1996) (Szary *et al.*, 1997) (Hamstra *et al.*, 1999) and with cytokine gene therapy (Consalvo *et al.*, 1995) (Mullen *et al.*, 1996) (Cao *et al.*, 1998, Nanni *et al.*, 1998). These results support investigation of the combination of AdCDA/5FC and cisplatin, evaluated by conventional colony forming assay.

RESULTS

Dose-Response to AdCDA + 5FC Suicide Gene Therapy

Prior to defining parameters for the AdCDA/5FC plus cisplatin combination treatment, it was necessary first to determine the dose-response relationship between 5FC and each of the MT1A2, HT29, and HT29p14 cell lines infected with the suicide gene vector AdCDA, or with the control vector AdDL70-3. In this way the sensitivity of each cell line, in the presence and the absence of the prodrug-converting enzyme, was established by clonogenic assay. Briefly, cells were infected with AdCDA or Add*l*70-3 (as described in Materials and Methods). Three hours later, cells were trypsinized and plated to low density and exposed to 5FC for 48 h and a further 3 days (HT29) to 5 days (MT1A2, HT29p14) in drug-free media. (Results for each cell line are shown graphically in figures 1A, 1B, and 1C, respectively and summarized in table 1). At this time, colonies were stained and counted. Percent survival for control and suicide gene vector infected cells was determined relative to the cells exposed to 0 mM 5FC for each virus, respectively.

Student's *t*-test indicates that the sensitivity of AdCDA infected cells to 5FC was significantly different from the sensitivity of the corresponding cells infected with AdDL70-3 control virus (at a 99.5% confidence interval; indicated as appropriate on figures). The LD_{50} values (defined as the concentration of 5FC necessary to effect a 50% reduction in clonogenic survival), were approximately 5mM, 0.5 mM and 25 mM for AdCDA infected MT1A2, HT29 and HT29p14 cells respectively.

Figure 3.1A. *Clonogenic survival of MT1A2 cells following treatment with AdCDA plus 5FC.* MT1A2 cells infected at an moi of 5 with AdDL70-3 (o) or AdCDA (•) exposed for 48 hours to concentrations of 5FC ranging from 0 to 25 mm followed by 5 days additional culture in regular growth media. Each datum point represents the mean $\pm$ SE three independent experiments performed in triplicate; * p $<<0.005$.

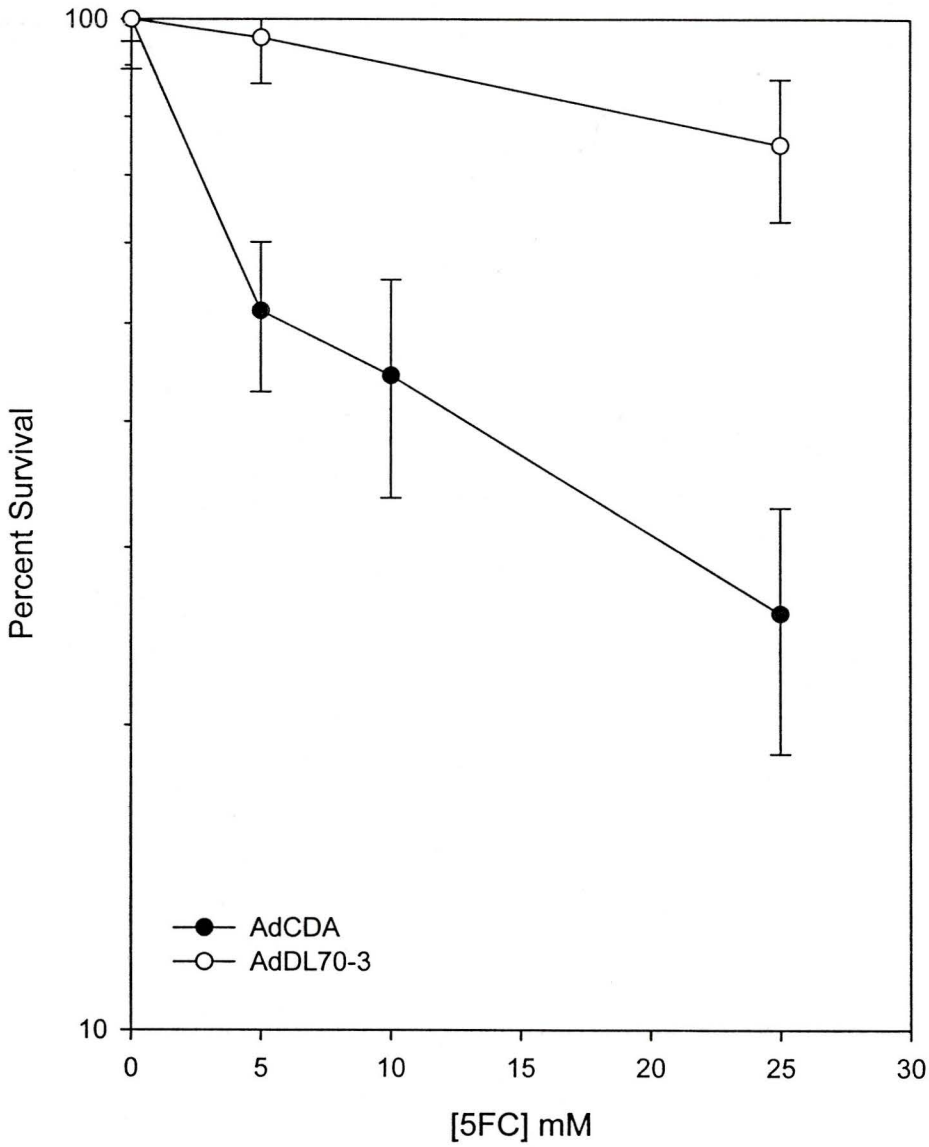

100
10
Percent Survival
0
5
10
15
20
25
30
[5FC] mM
AdCDA
AdDL70-3

Figure 3.1B. Clonogenic survival of HT29 cells following treatment with AdCDA plus 5FC. HT29 cells infected at an moi of 30 with AdDL70-3 (o) or AdCDA (•) exposed for 48 hours to concentrations of 5FC ranging from 0 to 25 mm followed by 3 days additional culture in regular growth media. Each datum point represents the mean $\pm$ SE three independent experiments performed in triplicate; $p << 0.005$.

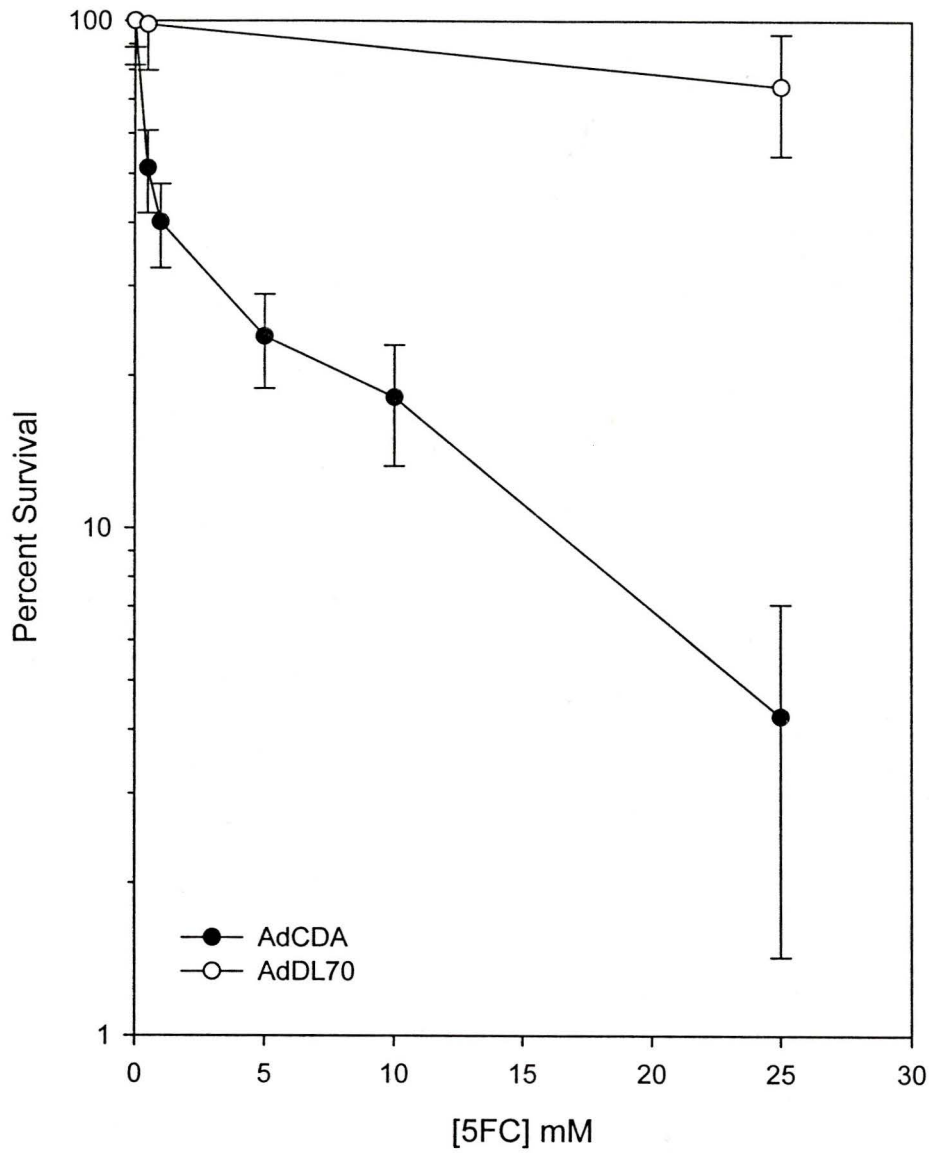

100
10
1
Percent Survival
0
5
10
15
20
25
30
[5FC] mM
AdCDA
AdDL70

Figure 3.1C. *Clonogenic survival of HT29p14 cells following treatment with AdCDA plus 5FC.* HT29p14 cells infected at an moi of 30 with AdDL70-3 (o) or AdCDA (•) exposed for 48 hours to concentrations of 5FC ranging from 0 to 25 mm followed by 5 days additional culture in regular growth media. Each datum point represents the mean ± SE three independent experiments performed in triplicate; $p < 0.005$.

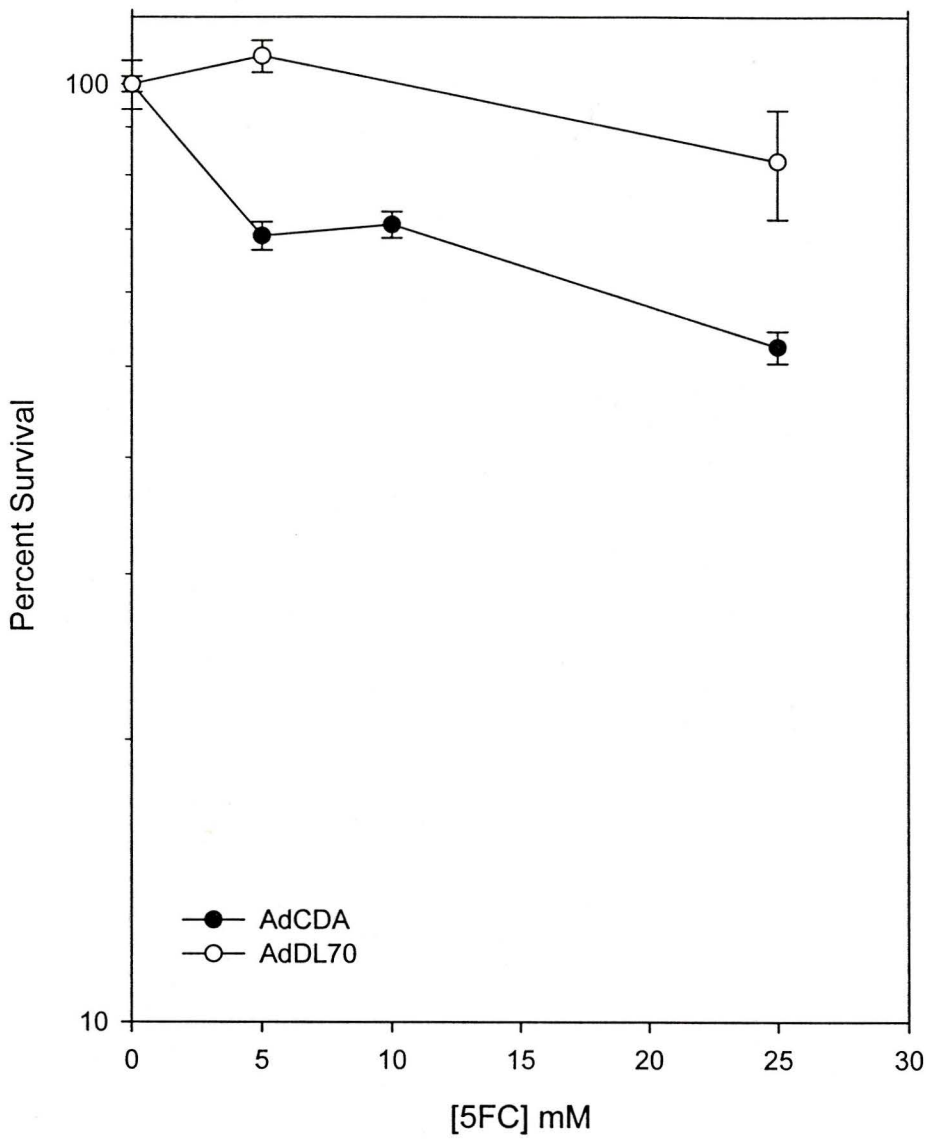

100
10
Percent Survival
0
5
10
15
20
25
30
[5FC] mM
AdCDA
AdDL70

Table 1: *Summary of Dose Responses To Single Modality Treatments*

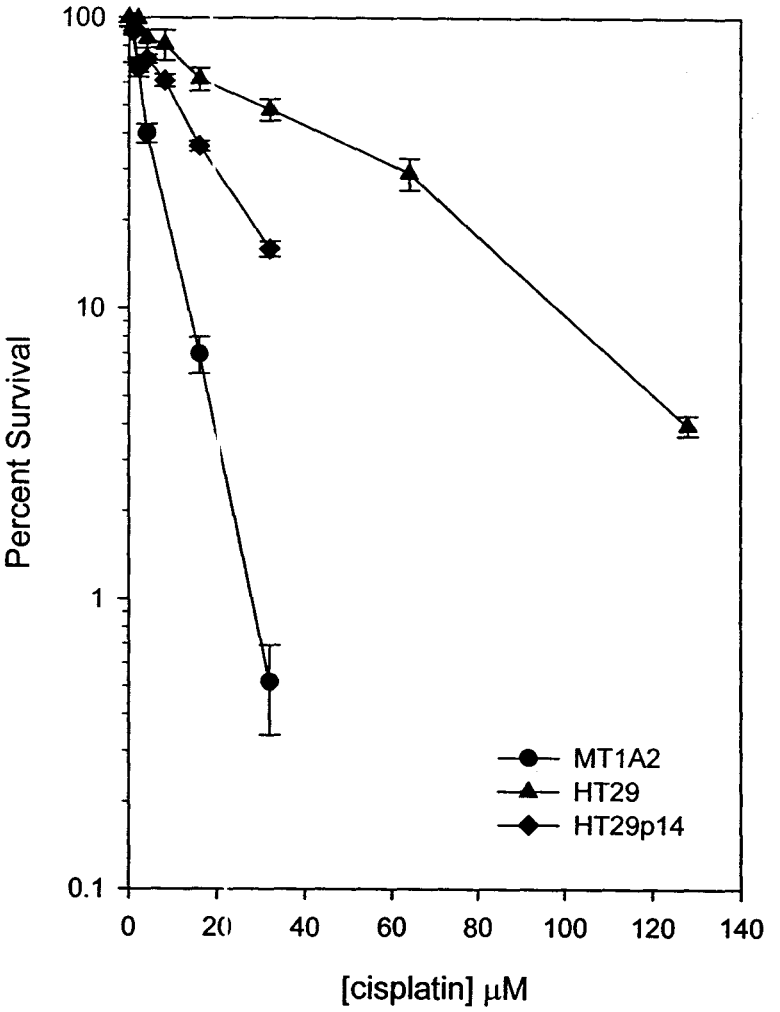
Cell line	Treatment	Dose	Percent Survival (±SE)*	Doses Chosen for Combination Treatment
MT1A2	AdCDA + 5FC	5 mM	51.5 (8.68)	5 mM 5FC + 4 and 16 μM cisplatin
	AdDL70-3 + 5FC	25 mM	75.15 (12.03)	
	cisplatin	4 μM	40 (3.04)	
HT29	AdCDA + 5FC	0.5 mM	51.3 (9.49)	0.5 mM 5FC 32, 64 μM cisplatin
	AdDL70-3 + 5FC	25 mM	74.3 (19.88)	
	cisplatin	32 μM	48.4 (4.17)	
HT29p14	AdCDA + 5FC	25 mM	52.49 (2.08)	15 mM 5FC 8, 32 μM cisplatin
	AdDL70-3 + 5FC	25 mM	82.8 (11.0)	
	cisplatin	8 μM	60.9 (3.02)	

* Percent survival for control and suicide gene vector infected cells was determined relative to infected cells exposed to 0 mM 5FC for each virus, independently.

Dose-Response to Cisplatin Chemotherapy

In addition to establishing an LD_{50} value for the response of each cell line to AdCDA/5FC suicide gene therapy, it was necessary also to establish the dose-response of each cell line to cisplatin alone, again using the clonogenic assay (Figure 3.2; Table 1). Briefly, 3 h after cells were plated to low density they were exposed to cisplatin for 1 h, then returned to culture in drug-free media for a further 5 days. At this time, colonies were stained and counted. Percent survival was determined relative to the cisplatin non-treated control cells. The LD_{50} cisplatin concentrations for MT1A2, HT29 and HT29p14 cells were approximately 3 μM, 32 μM and 12 μM respectively.

Figure 3.2. *Clonogenic survival of MT1A2, HT29, and HT29p14 cells exposed to cisplatin for 60 minutes.* MT1A2 (●), HT29(▲), and HT29p14 (◆)cells were plated at 100 cells per well and treated with cisplatin for 1 h at 3 h post-plating. Cells were cultured in regular growth media for a further 7 days. Each datum point represents the mean ± SE of three independent experiments each performed in triplicate.


100
10
1
0.1
Percent Survival
0
20
40
60
80
100
120
140
[cisplatin] μM
MT1A2
HT29
HT29p14

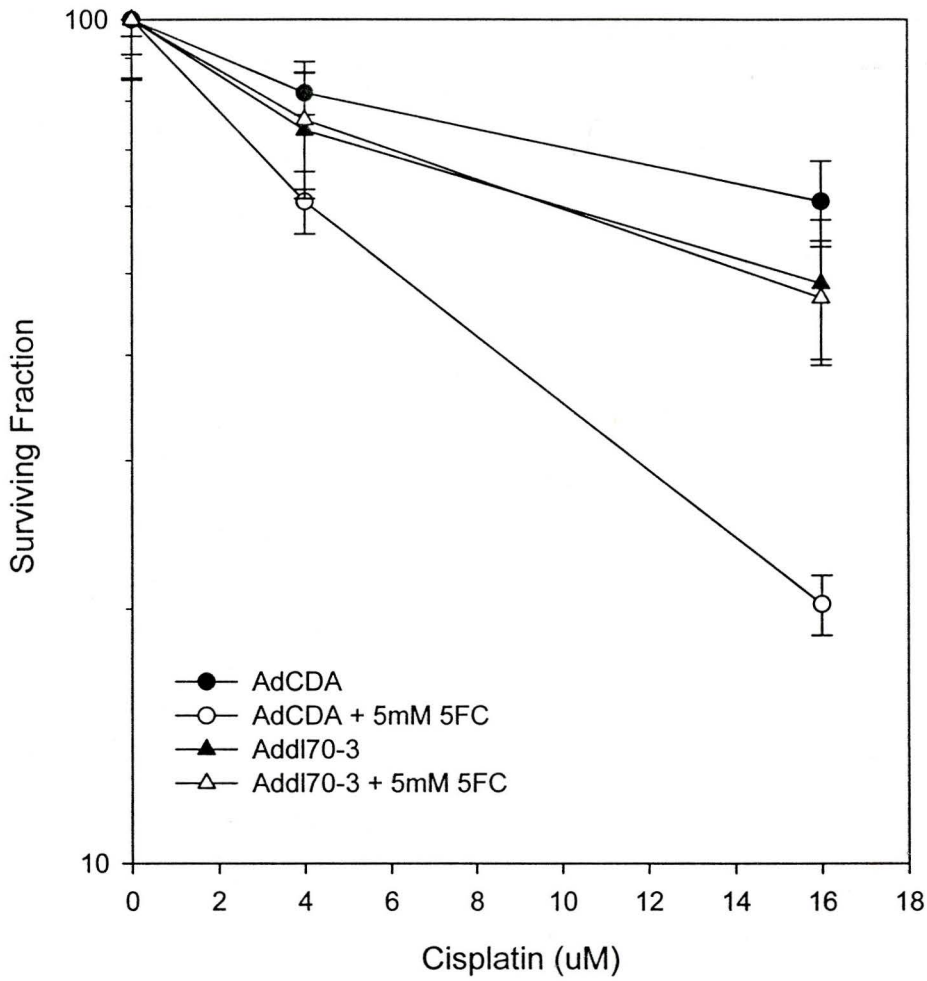
Dose-Response to AdCDA/5FC + Cisplatin Combination Treatment

The combination treatment consisted of pre-exposure of cells to either a single concentration of 5FC or drug-free media followed by exposure to two concentrations of cisplatin. Briefly, the colony forming assay was carried out as described above the the AdCDA/5FC alone treatment. However, after 48 h exposure to 5FC, cells were exposed to cisplatin for 1 h and then cultured in drug-free media for a further 3 days (HT29) or 5 days (MT1A2, HT29p14). Percent survival for control and suicide gene vector infected cells was determined relative to survival values obtained for cells exposed to 5FC or not exposed to 5FC, as appropriate. Curves representing cells exposed to 5FC are shown relative to the percent survival of infected cells cultured in the respective concentration of 5FC, but exposed to a concentration of 0 μM cisplatin.

For MT1A2 and HT29 cells the concentration of 5FC used was 5mM and 0.5 mM respectively and corresponded to the LD_{50} concentration for these two cell lines when infected with AdCDA. In the HT29p14 cells the LD_{50} concentration for AdCDA infected cells was more similar to that of AdDL70 infected cells, than it was for either MT1A2 or HT29 cells. A dose of 15mM 5FC was chosen for use in the combination since this concentration caused a substantial reduction in survival of AdCDA infected but not in AdDL70 infected HT29p14 cells. Results for MT1A2, HT29, and HT29p14 cells are shown in figures 3.3A, 3.3B, and 3.3C, respectively. All datum points represent the mean of three independent experiments, except as noted for the AdDL70-3 infected HT29p14 cells. Combination treatment curves have been corrected for the effect of 5FC. Error bars represent standard error of the mean. The statistical tests used were as described in the Materials and Methods.

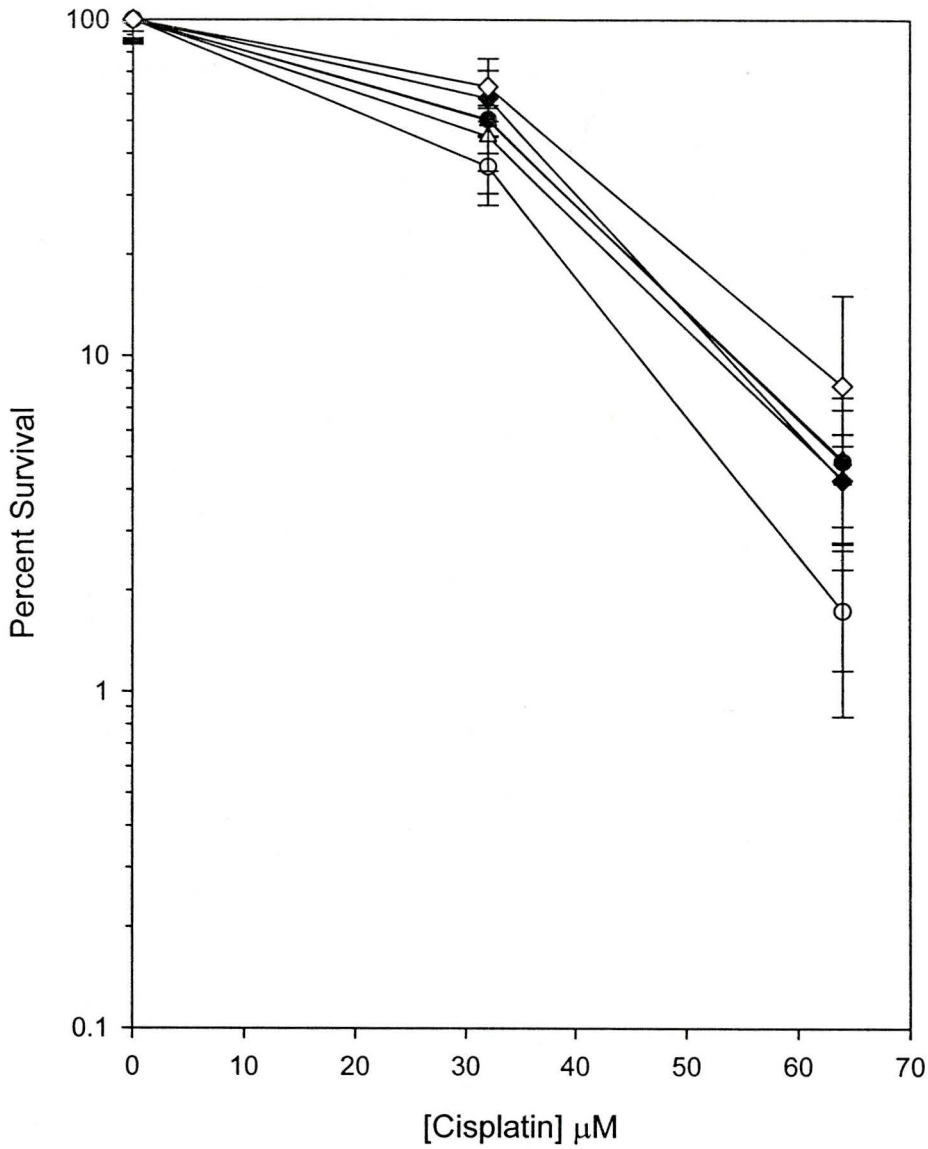
MT1A2 Cells (Figure 3.3A): As expected, survival of AdDL70-3 infected MT1A2 cells cultured in regular growth medium was not significantly different from survival of AdDL70-3 infected MT1A2 cells cultured in the presence of 5mM 5FC. The survival of AdCDA infected cells was greater for cells cultured in drug-free media compared to cells cultured in 5 mM 5FC. In this experiment, treatment of AdCDA infected cells with 4 μM and 16 μM cisplatin alone resulted in 82% and 62% survival, respectively. In contrast, the survival of AdCDA infected cells following treatment 5 mM 5FC and either 4 μM or 16 μM cisplatin following exposure to 5 mM 5FC was 61% and 20% respectively, indicating synergism of the combined cisplatin and AdCDA/5FC treatment (Table 2). The results of the t-test, P(t), for AdCDA infected cells showed that survival following combination treatment at 16 μM cisplatin was significantly different from survival following treatment with the same concentration of cisplatin alone (at a 95% confidence interval). The results of the cumulative χ^2 test, $P(\Sigma\chi^2)$, including survival values for both concentrations of cisplatin indicated that survival following the combination treatment was significantly different from treatment by cisplatin alone at a 99.5% confidence level. Complete results of the statistical analyses are presented in Table 3.

Figure 3.3A. *Clonogenic survival of MT1A2 cells exposed to the combination treatment of AdCDA/5FC + cisplatin.* Cells were uninfected or infected with AdDL70-3 or AdCDA at an MOI of 5 and pre-exposed to drug-free media (■, ▲, ●) or to 5 mM 5FC (□, △, ○) for 48 h followed by a 1 h exposure to cisplatin. Cells were cultured in drug-free media for an additional 5 days. Each datum point represents the mean ± SE for three independent experiments, conducted in triplicate.


100
10
Surviving Fraction
0
2
4
6
8
10
12
14
16
18
Cisplatin (uM)
AdCDA
AdCDA + 5mM 5FC
Addl70-3
Addl70-3 + 5mM 5FC

HT29 Cells (Figure 3.3B): Similar to MT1A2 results, survival of AdDL70-3 infected HT29 cells without 5FC was not significantly different from the survival of AdDL70-3 infected HT29 cells cultured in the presence of 0.5mM 5FC following exposure to cisplatin. The survival of AdCDA infected cells was greater for cells cultured in regular growth medium compared to cells cultured in the presence of 0.5 mM 5FC. Treatment of AdCDA infected with 32 μM or 64 μM cisplatin alone resulted in 81.8% and 60.9% survival, respectively. In contrast, the survival of AdCDA infected cells following treatment with 32 μM and 64 μM cisplatin and 0.5 mM 5FC was 60.8% and 20.3% respectively, suggesting a greater than additive effect of the combined cisplatin and AdCDA/5FC treatment (Table 2). The results of the t-test, P(t), for AdCDA infected cells showed that survival following combination treatment at 64 μM cisplatin was not significantly different from survival following treatment with the same concentration of cisplatin alone, as indicated by a p-value of only 0.092. However, the cumulative χ^2 test, $P(\Sigma\chi^2)$ summed over both cisplatin concentrations showed that survival following the combination treatment was significantly different from treatment with cisplatin alone, at a 97.5% confidence level. Complete results of statistical analyses are presented in Table 3.

Figure 3.3B. Clonogenic survival of HT29 cells exposed to the combination treatment of AdCDA/5FC + cisplatin Cells were uninfected or infected with AdDL70-3 or AdCDA at an MOI of 30 and pre-exposed to drug-free media (■, ▲, ●) or to 0.5 mM 5FC (□, △, ○) for 48 h followed by a 1 h exposure to cisplatin. Cells were cultured in drug-free media for an additional 3 days. Each datum point represents the mean ± SE for three independent experiments, conducted in triplicate.


100
10
1
0.1
Percent Survival
0
10
20
30
40
50
60
70
[Cisplatin] μM

HT29p14 Cells (Figure 3C): As observed with other cell lines, survival of AdDL70-3 infected HT29p14 cells without 5FC was not significantly different from the survival of AdDL70-3 infected HT29p14 cells cultured in the presence of 15mM 5FC following exposure to cisplatin . In contrast, the survival of AdCDA infected cells was greater for cells cultured in regular growth medium compared to cells cultured in the presence of 15 mM 5FC, following exposure to cisplatin. Treatment of AdCDA infected cells with 8 μM or 32 μM cisplatin alone resulted in 81% and 37% survival, respectively. In contrast, the survival of AdCDA infected cells following treatment with 8 μM and 32 μM cisplatin and 15 mM 5FC was 55% and 9% respectively, indicating synergism of the combined cisplatin and AdCDA/5FC treatment. Results of statistical analyses are presented in Table 3. The results of the t-test, P(t), for AdCDA infected cells showed that survival following combination treatment at 32 μM cisplatin was significantly different from survival following treatment with the same concentration of cisplatin alone (at a 99.5% confidence interval). The results of the cumulative χ^2 test, $P(\Sigma\chi^2)$, including survival values for both concentrations of cisplatin also indicated that survival following the combination treatment was significantly different from treatment by cisplatin alone at a 99.5% confidence level. Complete results of statistical analyses are presented in Table 3.

Figure 3.3C. *Clonogenic survival of HT29p14 cells exposed to the combination treatment of AdCDA/5FC + cisplatin.* Cells were uninfected or infected with AdDL70-3 or AdCDA at an MOI of 30 and pre-exposed to drug-free media (■, ▲, ●) or to 15 mM 5FC (□, △, ○) for 48 h followed by a 1 h exposure to cisplatin. Cells were cultured in drug-free media for an additional 5 days. Each datum point represents the mean ± SE for three independent experiments conducted in triplicate.

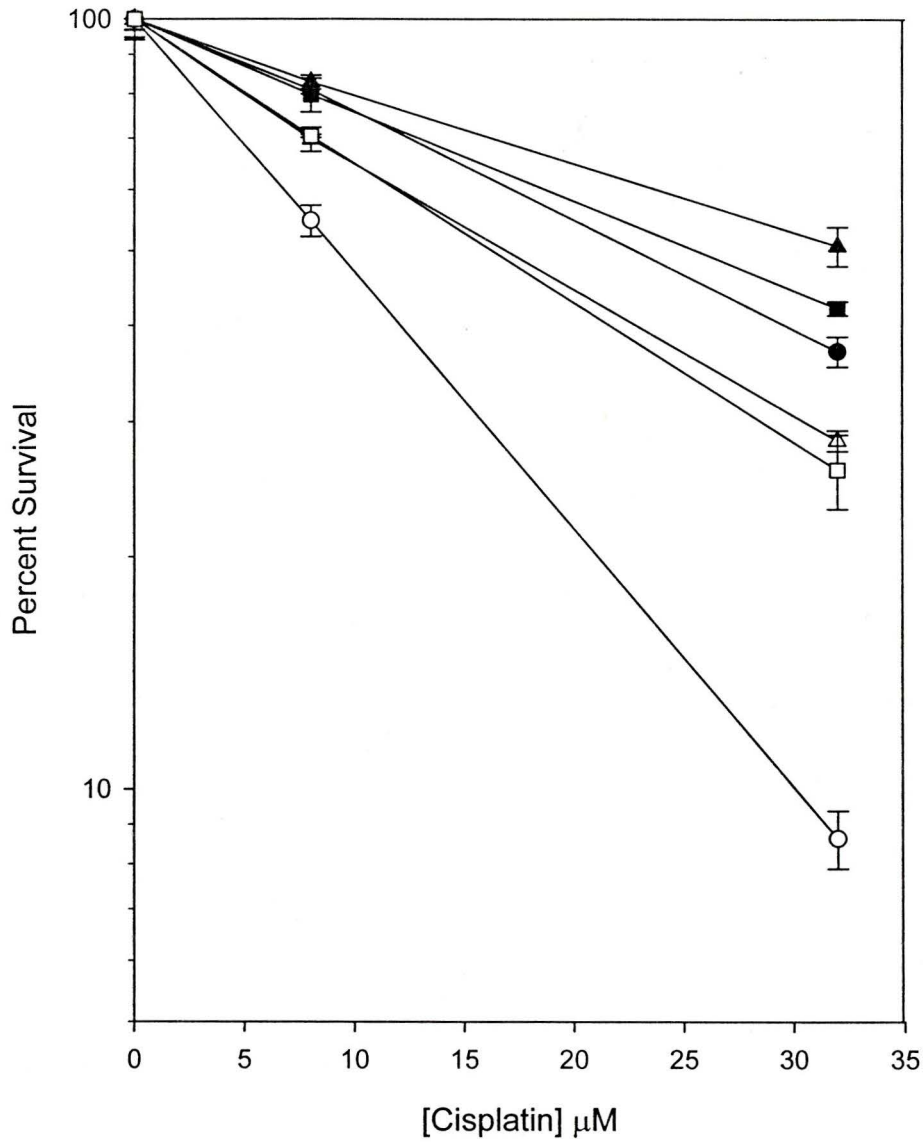

100
10
Percent Survival
0
5
10
15
20
25
30
35
[Cisplatin] μM

Table 2: Summary of Dose Response to Combination Treatment

			AdDL70-3 % Survival (± SE) *		AdCDA % Survival (± SE)	
	[5FC] mM	[Cisplatin] uM	-5FC	+5FC	-5FC	+5FC
MT1A2	5 mM	4	73.9 (12.54)	76.0 (13.1)	81.8 (4.74)	60.8 (5.16)
		16	48.8 (9.17)	46.8 (7.9)	60.9 (7.1)	20.3 (1.7)
HT29	0.5 mM	32	50.3 (5.4)	45.1 (9.5)	50.6 (20.1)	36.1 (8.6)
		64	4.9 (7.9)	4.3 (1.6)	4.9 (2.1)	1.7 (0.9)
HT29p14	15 mM	8	73.3 (6.2)	72.0 (7.5)	81.0 (1.0)	54.8 (7.7)
		32	54.7 (1.2)	50.3 (8.9)	37.1 (1.7)	8.6 (0.8)

- *Percent survival for control and suicide gene vector infected cells was determined relative to survival values obtained for cells exposed to 5FC or not exposed to 5FC, as appropriate. Curves representing cells exposed to 5FC have been are shown relative to the percent survival of infected cells exposed to 5FC, but exposed to 0 uM cisplatin.*

Table 3: Statistical Analysis of AdCDA/5FC + Cisplatin Combination Treatment

	[5-FC] (mM)	*	[Cisplatin] (μM)		P($\Sigma \chi^2$) 4, 16 (μM)
			4	*16*	
MT1A2	5	P(t)	0.47	0.05	
		χ^2	11.03	51.8	< 0.005
			32	*64*	
HT29	0.5	P(t)	0.271	0.092	
		χ^2	58.98	6.32	< 0.025
			8	*32*	
HT29p14	15	P(t)	0.020	0.005	
		χ^2	30.96	62.41	< 0.005

* Comparison of each point on the 5FC pretreatment curve with the corresponding point on the cisplatin-only treatment curve

In conclusion, the cell lines MT1A2 murine mammary carcinoma, HT29 human colon carcinoma, and HT29p14 photofrin resistant human colon carcinoma, were sensitive both to AdCDA/5FC treatment and to cisplatin treatment. Combined AdCDA/5FC and cisplatin treatment was at least additive in all cell lines tested. Statistical analysis suggests that the combination treatment may be synergistic in at least two of these cell lines, MT1A2 and HT29p14. Further study is required to elucidate the mechanism of cytotoxicity.

Discussion

The present work investigated the combination treatment of adenoviral-vector based cytosine deaminase plus 5-fluorocytosine suicide gene therapy (AdCDA/5FC) and cisplatin chemotherapy in MT1A2 murine mammary carcinoma cells, HT29 human colon adenocarcinoma cells and HT29p14 photofrin resistant human colon carcinoma cells. This investigation was suggested by observations, in pre-clinical and clinical research, that 5FU and cisplatin act synergistically when used in combination for the treatment of cancer. Prior to attempting the proposed combination, a dose-response to AdCDA/5FC and cisplatin, alone, was established for each cell line. Data from these preliminary experiments was used to choose suitable concentrations of both 5FC and cisplatin for incorporation into the combination treatment regimen. By clonogenic assay, the combination treatment was evaluated by comparing the survival of AdCDA infected cells exposed for 48 h to 5FC then for 1 h to cisplatin to the survival of cells exposed to drug-free media prior to the cisplatin treatment.

AdCDA/5FC Suicide Gene Therapy

It is widely accepted that expression of CDA confers lethal sensitivity to 5FC. This has been shown in a wide variety of mammalian cells using retroviral vectors (Mullen *et al.*, 1992) (Huber *et al.*, 1993, Huber *et al.*, 1994) (Mullen *et al.*, 1994) (Consalvo *et al.*, 1995) (Kuriyama *et al.*, 1998) (Pierrefite-Carle *et al.*, 1999) (Kuriyama *et al.*, 1999, Zhang *et al.*, 2000) vaccinia viral vectors (Gnant *et al.*, 1999) and adenoviral vectors (Hirschowitz *et al.*, 1995) (Dong *et al.*, 1996) (Ohwada *et al.*, 1996) (Li *et al.*, 1997) (Wolff *et al.*, 1998) (Shirakawa *et al.*, 1999) (Koyama *et al.*, 2000). The current work used Ad-vector mediated delivery of the CDA transgene to evaluate the effect of CDA/5FC gene therapy on the cell lines MT1A2, HT29, and HT29p14. Of the various available gene delivery vectors, Ad was chosen because of its ability to infect a wide variety of cell types with high efficiency. Also, it was shown previously that this adenovirus construct encoding CDA was capable of sensitizing MT1A2 cells to 5FC (Mary Hitt, 1999, upublished data). The effect of this AdCDA/5FC treatment was quantitated using a conventional colony forming assay. The colony forming assay measures the ability of treated cells to grow and divide *in vitro* with respect to untreated controls. It is well established that

this *in vitro* assay provides a realistic estimation of the same treatment, when administered *in vivo* (Roobol *et al.*, 1984, Shrivastav *et al.*, 1980).

The cytotoxicity of AdCDA/5FC was evaluated in MT1A2, HT29, and HT29p14 cells infected with the Ad*DL*70-3 control vector and the AdCDA suicide gene vector. As expected, in the absence of CDA, cells infected with the control vector Ad*DL*70-3 were not sensitive to low doses of 5FC. However, at the maximum concentrations of 5FC a slight reduction in survival of control vector infected cells was observed. Others have reported toxicity associated with high doses of 5FC *in vitro* despite the absence of the expression of CDA (Huber *et al.*, 1993). Similarly, it is often reported that patients experience toxicity following administration of 5FC. Toxic side-effects in patients are partly, but not completely, related to generation of 5FU by CDA expressing organisms in the natural gut flora (Pinedo & Peters, 1988). However, it has also been shown that intravenous preparations of 5FC administered to patients contain variable amounts of 5FU. The source of this contamination was linked to the use of 5FU as a precursor in the synthesis of 5FC (Cavrini *et al.*, 1991) and the formation of 5FU during the sterilization of 5FC at high temperatures (Vermes *et al.*, 1999). While the conditions necessary to generate 5FU through the heat-treatment of 5FC were not met in the present work, it is possible that 5FU was present as a contaminant in the raw 5FC. In *in vitro* cultures of CDA expressing cells, low levels of 5FU contamination would be masked by CDA-mediated conversion of 5FC to 5FU. However, in control vector infected cells contaminant 5FU could have caused the reduction in clonogenic survival observed at higher concentrations of 5FC.

When transduced with the AdCDA vector, MT1A2, HT29, and HT29p14 cells exhibited a dose-response to increasing concentrations 5FC. Each cell line was sensitive over a different range of 5FC concentrations, which could be accounted for by any of several factors. The amount of the CDA transgene product generated in each cell line is determined by the amount of vector administered, the transduction efficiency of each cell line and the strength of the transgene promoter within each cell type. Levels of CDA expression, in turn, influence the amount of 5FU generated, which ultimately determines clonogenic survival. The vector MOI used for each cell line was chosen such that CDA expression would not be a limiting factor in the overall cytotoxic effect of AdCDA/5FC treatment. For the MT1A2 cells, an MOI of 5 was thought to be sufficient since it is known that these cells are easily

transduced by Ad [Mary Hitt, unpublished data]. A preliminary AdCDA/5FC clonogenic assay in the HT29 cells compared an MOI of 10 to an MOI of 30 (data not shown). The reduction in survival associated with MOI 30 was attributed to increased generation of 5FU rather than toxicity of virus itself. Consequently the higher of the 2 MOI's was chosen for use in all subsequent experiments for both the HT29 and HT29p14 cell lines.

Levels of transgene expression depend on the amount of virus entering the cell. Transduction efficiency of cells by Ad is cell line specific and it is possible that murine cell have reduced levels of the Ad5 specific cell-surface receptors (Addison et al, 1997). While this may have contributed to differences in 5FC sensitivity of AdCDA transduced murine and human cells used in this work, such an effect was not evaluated empirically.

Levels of transgene expression are also affected by the choice of promoter. CDA expression was driven by the murine cytomegalovirus (MCMV) immediate early promoter. Addison *et al* (1997) demonstrated that the activity of the MCMV promoter is not species-specific and transgenes are expressed similarly well in most human and murine cells. Therefore, it is unlikely that differences in the strength of the MCMV promoter in murine and human cell lines contributed to observed differences in 5FC sensitivity of AdCDA infected cells. However, differences between the sensitivity to 5FC of AdCDA infected HT29 and HT29p14 cells may be related to transgene expression. This is discussed later in more detail.

In addition to the amount of CDA protein produced in the cell lines, differences in responses to AdCDA/5FC treatment were also related to the intrinsic sensitivity of each cell line to 5FU. 5FU sensitivity is mediated by a complex interaction of enzymes and other cellular molecules, all of which vary between cell lines and cell types. A more complete description of these factors is presented in Chapter 1. Despite this information, it remains difficult to ascertain exactly why the MT1A2, HT29, and HT29p14 cell lines responded differently to AdCDA/5FC therapy. For the most part, the specific mechanisms by which 5FU exerts cytotoxic effects in each of these particular cell lines have not been characterized, and no such investigations were carried out in the current work. It can be said, however, that while sensitivity is highly cell-specific, it does not appear to be species-specific (Peters *et al.*, 1986).

Of note, however, is that HT29 cells were significantly more sensitive to treatment with AdCDA/5FC than HT29p14 cells. Presumably, this is a consequence of the biologic changes associated with the establishment of resistance to Photofrin-mediate PDT in HT29p14 cells, although these changes have not yet been characterized (Singh *et al.*, 2001). It can be hypothesized that these changes affected the intracellular concentrations of enzymes or molecules involved in 5FU cytotoxicity. Alternatively, levels of CDA expressed within the HT29p14 cells may have been negatively affected.

Even though the responses of these two cell lines to treatment by AdCDA/5FC were different, HT29 and HT29p14 cells exhibited approximately the same dose response to a 48 h exposure to 5FU (please refer to Chapter 4 Results, Fig 6). Exposure of cells to 5FU for 48 h may not be the same as exposure of CDA-expressing cells to 5FC for 48 h. The suicide gene therapy protocol requires the expression of CDA and the conversion of 5FC to 5FU prior to the generation of cytotoxic 5FU metabolites FdUMP and FUTP. The time requirement of these events could have detracted from the net amount of time that 5FU was actually present within the cell. It has been shown that the mechanism of 5FU cytotoxicity can depend on the dosage and schedule of administration of 5FU in *in vitro* culture systems (Calabro-Jones *et al.*, 1982). This is also true of 5FU used in the clinic (Macdonald, 1999). Thus, AdCDA/5FC may constitute a short-term gradient exposure whereas direct administration of 5FC may constitute as a long term continuous exposure. Sensitivity to short-term exposures has been related to incorporation of FUTP into RNA, while sensitivity to long term exposures seems more dependent upon TS inhibition (Aschele *et al.*, 1998). Therefore, the difference observed between HT29 and HT29p14 cells exposed to AdCDA/5FC but not when exposed to 5FU could be related to the fact that these two experiments may have constituted different schedules of administration and that these cells do not react in the same way to short and long term exposures to 5FU.

In addition to changes in the sensitivity of cells to 5FU, it is also possible that the establishment of PDT resistance negatively affected the levels of CDA expression achieved in HT29p14 cells. It has been shown that conversion of 5FC to 5FU by CDA is directly proportional to vector MOI (Koyama *et al.*, 2000). Even though HT29 and HT29p14 cells were both infected with AdCDA at an MOI of 30 they may not be transduced by Ad equally due perhaps to a change in the Ad receptor status of HT29p14 cells. Alternatively, the alterations in cell biology associated with photofrin resistance may

have negatively affected the levels of CDA expression mediated by the MCMV promoter within the HT29p14 cells.

Cisplatin Chemotherapy

Each of the cell lines HT29, HT29p14, and MT1A2 exhibited a dose-response to increasing concentrations of cisplatin. However, the sensitivity to cisplatin of each cell line, relative to the others, was dependent on the experimental protocol used. In preliminary experiments, cells were exposed to cisplatin 3 h after being plated to low density. In the combination experiments cisplatin exposure was carried out 48 h after cells were plated to low density. Results of the preliminary cisplatin experiments will be explained first, followed by discussion of the results obtained from the cisplatin-only treated groups in the AdCDA/5FC + cisplatin combination experiment.

The murine cell line MT1A2 was more sensitive to cisplatin than both the HT29 and HT29p14 cells, according to the LD_{50} values observed for each cell line in response to cisplatin. There are several possible explanations for this. In general, murine cells are more sensitive to cisplatin than human cells (Rosenberg, 1985). Furthermore, it has been shown that colorectal carcinomas are inherently more resistant to cisplatin than other tumor types (Perez, 1998). HT29, and presumably HT29p14, cells are deficient in the tumour suppressor gene *p53* (Peters *et al.*, 2000b), whereas MT1A2 cells express wild-type p53 protein (Putzer *et al.*, 1997). Activation of *p53* by cisplatin damaged DNA can lead to cell cycle arrest or ultimately to cell suicide through the induction of apoptosis (Gonzalez *et al.*, 2001). Thus, the loss of p53 function in the HT29 and HT29p14 cells could explain, in part, their increased resistance to cisplatin.

The HT29p14 cells exhibited increased sensitivity to cisplatin compared to the parental HT29 cells. This result contrasted with previous studies in human ovarian carcinoma (2008) cells and radiation induced fibrosarcoma (RIF-8A) cells where resistance to PDT was associated with cross-resistance to cisplatin (Moorehead *et al.*, 1994). Since HT29p14 cells were not cross-resistant to cisplatin, nor have they been characterized with respect to their response to other cytotoxic drugs, there is no existing data to contribute to an explanation of the difference in cisplatin sensitivity between HT29

and HT29p14 cells. Further investigation is required to enable a better understanding of the molecular determinants of sensitivity and resistance to cisplatin and photofrin in these two cell lines.

The sensitivity of cells to cisplatin observed in the combination experiment was different from that of the preliminary experiments. In the combination experiments MT1A2 cells and HT29p14 cells were more resistant to cisplatin, than in the preliminary experiment, while the HT29 cells were more sensitive. For each cell line, in the combination study, the uninfected and Ad*DL*70-3 infected cells cultured in media with or without 5FC, and the AdCDA infected cells cultured in media without 5FC all exhibited the same dose-response to cisplatin. This suggests that the difference in cisplatin sensitivity observed between the preliminary and combination treatments could be related to the conditions under which cells were exposed to cisplatin. In preliminary experiments confluent cells were trypsinized, plated to low density and exposed to cisplatin 3 h later. Cisplatin would have been administered to single cells. In the combination experiments, cells were exposed to cisplatin after having been in culture for 2 days and probably would have been present as colonies consisting of 2 to 4 cells.

At 3 hours post-plating, cells would not yet have divided and would still be present as isolated, single cells, rather than a colony of more than one cell. Thus, the elimination of what would become a colony would require death of a single cell. In contrast, 48 hours post-plating, surviving cells likely would have undergone 2 to 3 complete cell cycles, given variations in cell cycle time between 16 and 24 hours. Cisplatin would have been administered to small colonies of 2 to 4 cells, not to single cells as above. It follows that complete elimination of a colony, from the point of view of quantitation at the end of the clonogenic assay, would require killing of all cells in the colony by cisplatin, rather than only a single cell. Were only a fraction of the cells in any given colony eliminated, the remaining cells could have been capable of further division resulting in a colony that would be included when the colonies were counted.

The exposure of a single, isolated cell to cisplatin is likely to have a different effect than exposure to several cells in contact with each other. Contact between cells facilitates intracellular communication, often via gap junctions, which are transmembrane channels connecting the cytoplasm of adjacent cells (Holder *et al.*, 1993). The exchange of ions, metabolites and other cellular compounds through gap junctions provides a mechanism by which adjacent cells maintain homeostasis and sick

cells can be nurtured by cells adjacent to them. In addition, the harmful effects of chemicals, such as anticancer agents, can be diluted by their dispersal via gap junction communication from one cell to several cells. Gap junction communication could explain the decreased effect of cisplatin on several cells compared to its effect on single cells, as evident from the results of cisplatin exposure in the combination and preliminary assays, respectively. Furthermore, since the number of gap junction channels formed between cells is cell type specific, the increased resistance to cisplatin observed in MT1A2 and HT29p14 cells contrasted to the decreased resistance observed in HT29 cells, may reflect quantitative differences in the regarding the number of gap junctions formed by these cell lines. Quantitation of the extent of gap junctions formed in each of the cell lines would be required to further evaluate this hypothesis.

AdCDA/5FC & Cisplatin Combination Treatment

A substantial amount of pre-clinical data, as well as some clinical data demonstrate the synergistic interaction between 5FU and cisplatin. The current work investigated the combination of AdCDA/5FC suicide gene therapy plus cisplatin chemotherapy in murine (MT1A2) and human (HT29 and HT29p14) cell lines. Briefly, the combination treatment was a clonogenic assay in which AdCDA infected cells were exposed for 48 h to a single dose of 5FC, or to drug-free media, followed immediately by a 1 h exposure to cisplatin. For each cell line, the combination was tested using a low and a high concentration of cisplatin. Cells were cultured for a total of 5 to 7 days after which time colonies were counted.

There are several methods used to evaluate whether the effect of a combination treatment is antagonistic, additive, or synergistic. The 'gold standard' of these is the isobologram (Peters *et al.*, 2000a). Since the current data were not suitable for analysis by isobologram, a statistical analysis was conducted (Caney *et al.*, 1999). The Student *t*-test compared the combination treatment to the cisplatin-alone treatment using survival values corresponding to the higher dose of cisplatin. A greater than additive effect of the combination was shown in the MT1A2 and HT29p14 cells, but not in the HT29 cells. However, analysis by $\Sigma(\chi^2)$, which included survival values obtained at both doses of cisplatin, showed that the effect of the combination treatment was greater than additive in each of MT1A2, HT29,

and HT29p14 cell lines. In essence, this analysis demonstrated a significant difference between the survival curves for treatment with the combination and by cisplatin-alone (Bevington, 1969).

For the HT29 cells, the absence of a statistically significant difference between combination and cisplatin-alone treatments, as shown by the *t*-test could be explained in part by the following. First of all, the survival data for HT29 cells treated with the AdCDA/5FC + cisplatin combination is associated with standard error values that were large enough to negate the presence of a statistically significant difference between the two means. Secondly, other work has shown 5FU-cisplatin synergism depends on the dose of each drug as well as the order in which they are administered (Scanlon *et al.*, 1986) (Johnston *et al.*, 1996, Shirasaka *et al.*, 1993). Therefore it could be that the conditions of the current experiment were not ideal to facilitate synergism between CDA-generated 5FU and cisplatin. It is recommended that the combination treatment in HT29 cells be repeated, using the existing protocol as well as others in which the parameters for dosing and schedule of administration of the suicide gene therapy and cisplatin chemotherapy are varied. This would generate a larger data set, for a greater variety of combinations such that the *t*-test could be repeated to better clarify whether there is a significant difference between the survival means.

It is important to note that the protocol for these experiments is such that cisplatin treatment of AdCDA infected cells exposed to 5FC may not be equivalent to cisplatin treatment of control vector infected cells, or CDA infected cells not exposed to 5FC. CDA expressing cells exposed to 5FC for 2 days are less likely to have divided as often compared to the other cell lines, if they divided at all. Thus, it would be expected that a greater proportion of cells would have been present as single cells and the effect of cisplatin may have been amplified compared to its effect on cells present in colonies of 2 to 4 cells, as described above. The extent to which this aspect of this protocol interfered with obtaining a true evaluation of the effect of combined AdCDA + cisplatin should be addressed. One possibility would be to administer both treatments simultaneously. however, previous reports indicate that cisplatin and 5FU synergism is dependent on the order in which drugs are administered. So, although concurrent administration is a simple solution to this problem, it may be a dosing schedule that does not facilitate synergism. Other protocols would have to be devised that acknowledge both schedule dependency and the problem of treating single cells versus colonies that is described above.

Currently, there is no existing precedent for the combination of AdCDA/5FC suicide gene therapy and cisplatin chemotherapy. However, several groups have investigated the use of CDA/5FC suicide gene therapy with other cancer treatments, such as radiation and cytokine gene therapy.

CDA/5FC & Radiation Therapy

The use of 5FU as a radiosensitizing agent has been established for the treatment of several human malignancies, most notably squamous cell cancers of the head and neck (Stupp & Vokes, 1995) and gastrointestinal cancers (Arcangeli *et al.*, 1995). The benefit of combined 5FU and radiation over radiation alone can be explained by the potentiation of radiation induced DNA damage by 5FU (Lawrence *et al.*, 1994), the elimination by 5FU of radioresistant cells (Hanna *et al.*, 1997), the potentiation of 5FU induced cytotoxicity by radiation (Byfield, 1989), and the prolongation of 5FU retention in tumours following radiation pre-treatment (Blackstock *et al.*, 1996).

Extending from this work, several groups have substituted CDA/5FC suicide gene therapy for the direct administration of 5FU in chemoradiation treatment regimens. Not only was it shown that this treatment is as effective as 5FU + radiation, but also that it introduces benefits that are specific to the use of an enzyme/prodrug system (Khil *et al.*, 1996) (Hanna *et al.*, 1997, Szary *et al.*, 1997) (Hamstra *et al.*, 1999). For example, high doses of 5FC can be administered systemically to the patient that exceed the maximum allowable doses of 5FU. Conversion by CDA within the tumour facilitates correspondingly high levels of 5FU within the tumour that are otherwise unattainable because of the severe, dose-limiting toxicity associated with the direct administration of 5FU (Huber *et al.*, 1993). In addition, it is possible that the immunogenicity of the suicide gene product itself (as described in Chapter 1) confers additional anti-tumour effects related to the expression of the transgene. Hamstra et al, 1999, using comparable doses of 5FC and 5FU, showed that CDA/5FC and radiation caused a synergistic decrease in tumour growth and significantly increased survival in animals. The same result was not achieved using 5FU and radiation. Since this was evaluated in mice bearing syngeneic tumours it is possible that increased survival in animals exposed to the suicide gene therapy was related in part to the immunostimulatory properties of the transgene itself.

Radiation has also been used in combination with gene therapy protocols as a means of controlling gene expression. Several radiation inducible promoters, such as the CMV promoter (Francis, 2000), and early growth response (Egr-1) promoter (Datta *et al.*, 1992) have been identified. Hallahan et al, 1995 tested the combination of ionizing radiation and TNF-α under the Egr-1 promoter. Although TNF-α is a radiosensitizing cytokine, its application in this context is limited because of the severe systemic toxicity associated with TNF-α. An Ad-5 vector containing TNF-α under the Egr-1 promoter allowed both temporal and spatial control of gene expression. This resulted in a greater anti-tumour effect than was achieved using radiation alone and was not associated with significant toxicity.

CDA/5FC & Cytokine Gene Therapy

The rationale for combining cytokine gene therapy with CDA/5FC suicide gene therapy reflects several aspects of cancer gene therapy. Cancer is notorious for its ability to evade detection by the immune system. The therapeutic advantages of immunostimulation as a part of cancer treatment regimens is well-established and the subject of intense investigation. Immunotherapy, as an emerging cancer treatment modality, can be administered in the form of cytokine gene therapy rather than by the direct administration of cytokines, which has been associated with significant toxicity (reviewed in Hitt, 2000). Combining cytokine gene therapy with CDA/5FC suicide gene therapy accommodates for the fact that CDA/5FC does not always lead to complete tumour regression or protect against tumour re-growth (Consalvo *et al.*, 1995, Nanni *et al.*, 1998), and capitalizes on the anti-cancer effects of cytokine induced immunostimulation. Cao et al, 1998 demonstrated that the concurrent administration of AdCDA/5FC + AdGM-CSF against B16/F10 cell tumours *in vivo* had a greater than additive inhibition of tumour growth than either treatment alone. It has been suggested that the cellular debris generated by AdCDA/5FC mediated tumour cell apoptosis was taken up by GM-CSF activated dendritic cells and tumour associated antigens were then presented to the immune system, successfully inducing a potent anti-tumour immune response. Ju et al, 1998 were similarly successful in combining AdCDA/5FC and AdIL-2, citing increased infiltration of CD4+ and CD8+ T cells into the tumour and increased specific anti-tumour CTL activity. Using retroviral vectors, tumour cells engineered to co-express CDA with either IL-6 (Mullen *et al.*, 1996) or IFN-γ (Nanni *et al.*, 1998), 1998) were effective in causing

regression of tumours with or without the addition of the pro-drug 5FC. In both cases the immunogenicity of the transgene itself was cited as being essential to the overall immunostimulatory effect of the combined therapy that successfully caused tumour regression.

Conclusion

Pursuit of the combination of AdCDA/5FC suicide gene therapy and cisplatin chemotherapy is supported in several ways. First of all, as described in Chapter 1, the combination of 5FU and cisplatin appears to be synergistic in a variety of tumour cell lines, *in vitro* and *in vivo*. Synergism, however, seems to be dependent on the schedule of administration of the two agents in a cell specific manner. The efficacy of AdCDA/5FC has been demonstrated repeatedly in a variety of tumour types *in vitro* and *in vivo*. Combinations of AdCDA/5FC with radiation or cytokine gene therapy have also produced encouraging results regarding the use of this suicide gene therapy in combination with other agents.

The current work supports further investigation of AdCDA/5FC and cisplatin as a combination anti-cancer treatment. Additional study is required to provide a clearer understanding of this treatment. First of all experimental protocols need to be refined so that a stricter evaluation of synergism can be conducted. *In vitro,* the sequence dependence of the combination and biochemical mechanisms of interaction of these two therapies should also be investigated. Finally, the combination should be transferred to mouse models so the efficacy of this combination can be evaluated *in vivo*.

CHAPTER 4

ADCDA/5FC SUICIDE GENE THERAPY OF B16/F10 CELLS

Introduction

The highly metastatic murine melanoma B16/F10 cells, herein referred to as F10 cells, are used widely in cancer research. Their inclusion in the investigation of the combination AdDCA/5FC + cisplatin treatment was originally intended to provide another murine tumour model, in addition to the MT1A2 cells of the polyomavirus middle-T (PyMT) murine model of breast cancer and the two human colorectal carcinoma cells lines, HT29 and HT29p14. It is well understood that 5FU and cisplatin, administered alone or in combination, exhibit a high degree of cell-type specificity. 5FU cytotoxicity is dependent on a host of enzymes whose intracellular concentrations are cell-type specific. Similarly, cisplatin cytotoxicity depends on the extent of damage it incurs to DNA. Cells more resistant to cisplatin are either better able to repair the damage, or capable of progressing through the cell cycle despite the presence of cisplatin induced lesions in the DNA or take up less cisplatin. It followed, then, that the inclusion of many cell lines in these initial investigations of the AdCDA/5FC + cisplatin combination experiments was warranted since the characteristics of each cell line could be compared with their demonstrated response to the novel treatment. In addition, the F10 cells are transplantable in vivo and are used extensively for furthering our understanding of tumour biology and screening novel anti-cancer treatments. The inclusion of F10 cells into this particular project would be relevant not only to the immediate work, but also to a greater body of knowledge regarding this particular cell line.

Preliminary results of the investigation of AdCDA/5FC + cisplatin in the F10 cells indicated that these cells, like the MT1A2, HT29, and HT29p14 cells, were sensitive to each treatment alone and it appeared that the combination a greater than additive effect. However, upon repetition of the preliminary experiments, the original data was not reproducible. In these later experiments the dose response of the F10 cells to AdCDA/5FC treatment was entirely unrepresentative of the results obtained from the original experiments. Specifically, at first it appeared as though the LD_{50} concentration of 5FC

for AdCDA infected F10 cells was approximately 15 mM. Subsequent experiments indicated that the LD_{100} was in fact less than 5 mM. Several variables were deemed critical to any investigation designed to determine why repeatable results were not obtained using the F10 cells. Theses were: the difference between different preparations of 5FC to which the F10 cells were exposed, the sensitivity of F10 cells to 5FU, the enzymatic activity of CDA in AdCDA infected F10 cells, and the levels of transduction achieved by Ad vectors in F10 cells. The following is an investigation of the discrepancy of response exhibited by F10 cells to treatment with AdCDA/5FC.

Results:

AdCDA/5FC, Cisplatin, and Combination treatments

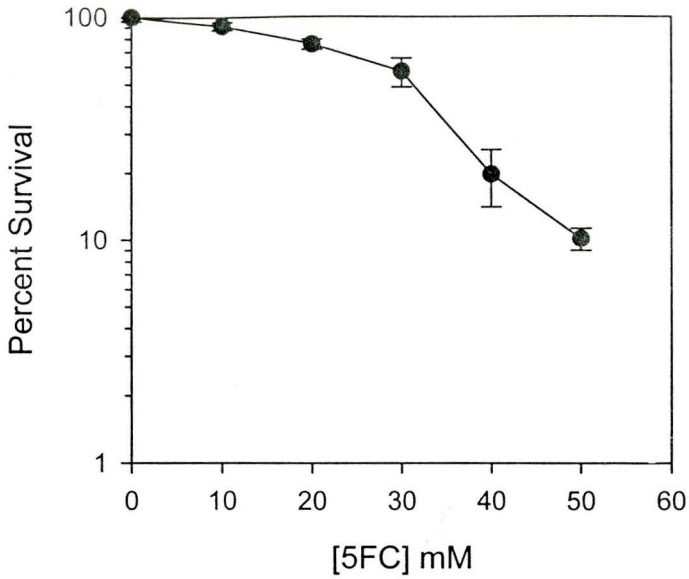
The same sequence of experiments carried out in the MT1A2, HT29, and HT29p14 cells (AdCDA/5FC, cisplatin, combination treatment) were also carried out in the F10 cells. The only exception was the exclusion of the AdDL70-3 control vector infected cells in CDA/5FC alone and CDA/5FC + cisplatin combination experiments since this was included as a control only in later experiments. The results of these early experiments showed that F10 cells transduced with the AdCDA suicide gene vector demonstrated a dose-response to 5FC with an LD_{50} of approximately 30 mM (Figure 4.1A). F10 cells exhibited a dose-response to cisplatin with an LD_{50} of approximately 10 μM (Figure 4.1B). Finally, initial CDA/5FC + cisplatin treatment suggested that AdCDA infected cells pretreated with 15 mM 5FC followed by exposure to cisplatin had a greater than additive effect on clonogenic survival (Figure 4.1C). Survival is expressed relative to the AdCDA infected cells exposed to 0 mM 5FC for 48 h.

Figure 4.1

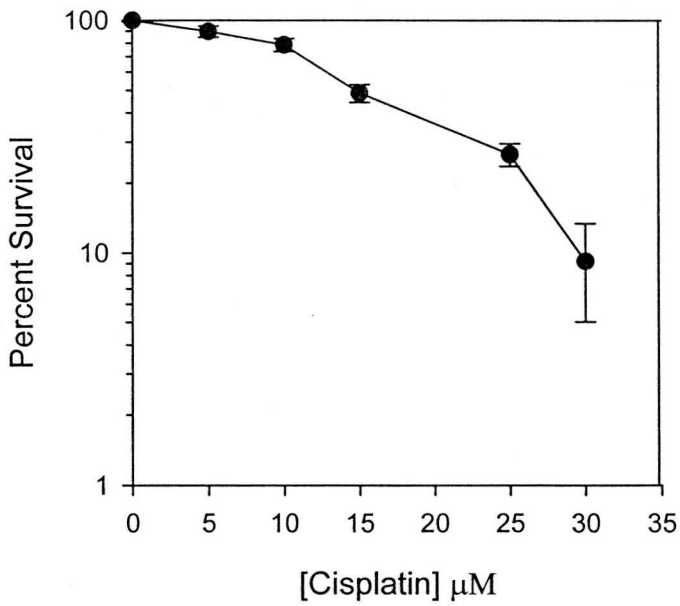
(A) Clonogenic survival of F10 cells following treatment with AdCDA plus 5FC. F10 cells infected with AdCDA at an MOI of 50 were exposed for 48 h to concentrations of 5FC ranging from 0 to 50 mM followed by 5 days additional culture in regular growth media. Each datum point represents the mean ± SE for one experiment performed in triplicate.

(B) Clonogenic survival of F10 cells exposed to cisplatin for 60 minutes. F10 cells were plated at 50 cells per well and treated with cisplatin for 1 h at 3 h post-plating. Cells were cultured in regular growth media for a further 7 days. Each datum point represents the mean ± SE for one experiment performed in triplicate.

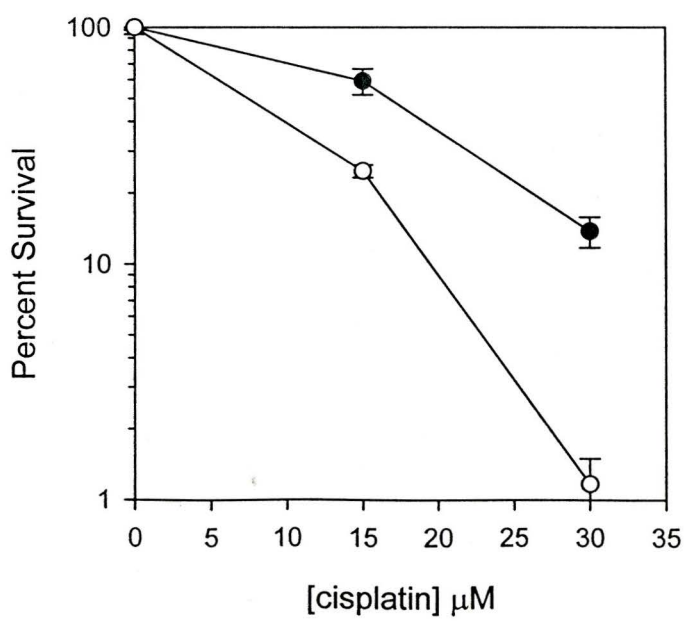
(C) Clonogenic survival of F10s exposed to the combination treatment of AdCDA/5FC + cisplatin. Cells were infected at an MOI of 50 with AdCDA and pre-exposed to drug-free media (●) or to 15 mM 5FC (○) for 48 h followed by a 1 h exposure to cisplatin. Cells were cultured in drug-free media for an additional 3 days. Each datum point represents the mean ± SE for a single experiment conducted in triplicate.


100
10
1
Percent Survival
0
10
20
30
40
50
60
[5FC] mM

A


100
10
1
Percent Survival
0
5
10
15
20
25
30
35
[Cisplatin] μM

B


100
10
1
Percent Survival
0
5
10
15
20
25
30
35
[cisplatin] μM

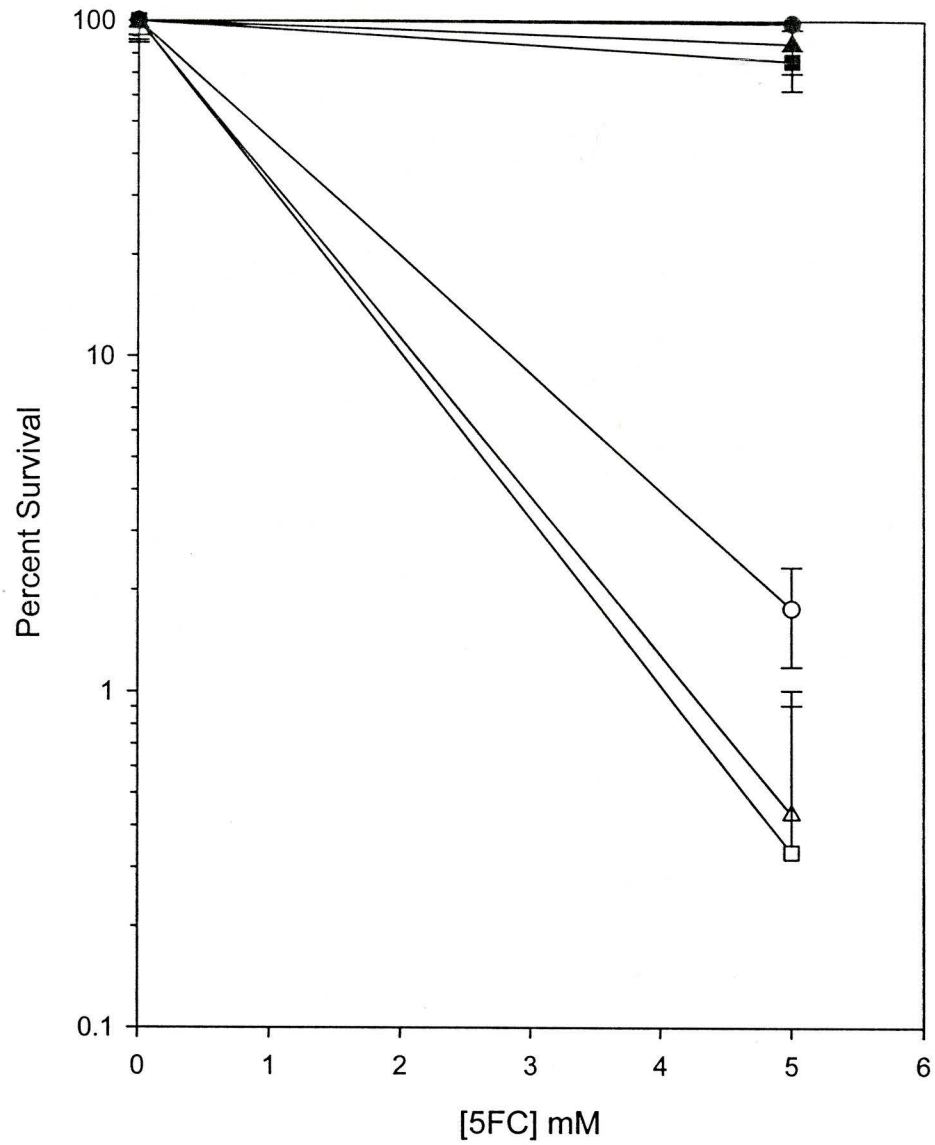
C

Variation in drug toxicity

Attempts to repeat the above experiments using the F10 cells, with inclusion of the appropriate controls, revealed that the dose-response of F10 cells infected with AdCDA was not reproducible from one experiment to the next, nor was there a difference between the dose response to 5FC of suicide gene and control vector infected cells. Whereas the original dose response (Figure 4.1A) demonstrated an LD50 of approximately 30 mM, a later experiment showed 100% killing at a dose less than 5 mM for cells infected with AdCDA and AdDL70-3 (data not shown).

It has been suggested that different preparations of 5FC may be differentially toxic even when prepared at the same concentration (M. Hitt and A. Schuh, unpublished observations). To address whether the difference observed in F10 sensitivity to 5FC in different experiments could be accounted for by differences between the 5FC preparations, a clonogenic assay was used to compare two different preparations of 5FC (Figure 4.2). F10 cells infected with AdCDA and AdDL70-3 were exposed to 5FC obtained from two different sources. A research-grade preparation was obtained from Sigma Chemical Supply Company and a pharmaceutical grade preparation was obtained from ICN Canada. Both AdCDA and AdDL70-3 infected F10 cells exposed to Sigma 5FC exhibited a markedly different dose response than similarly infected cells exposed to ICN 5FC. It appeared that the preparation of 5FC itself, rather than the presence of the prodrug-converting enzyme, was responsible for the observed cytotoxicity at a concentration of 5 mM.

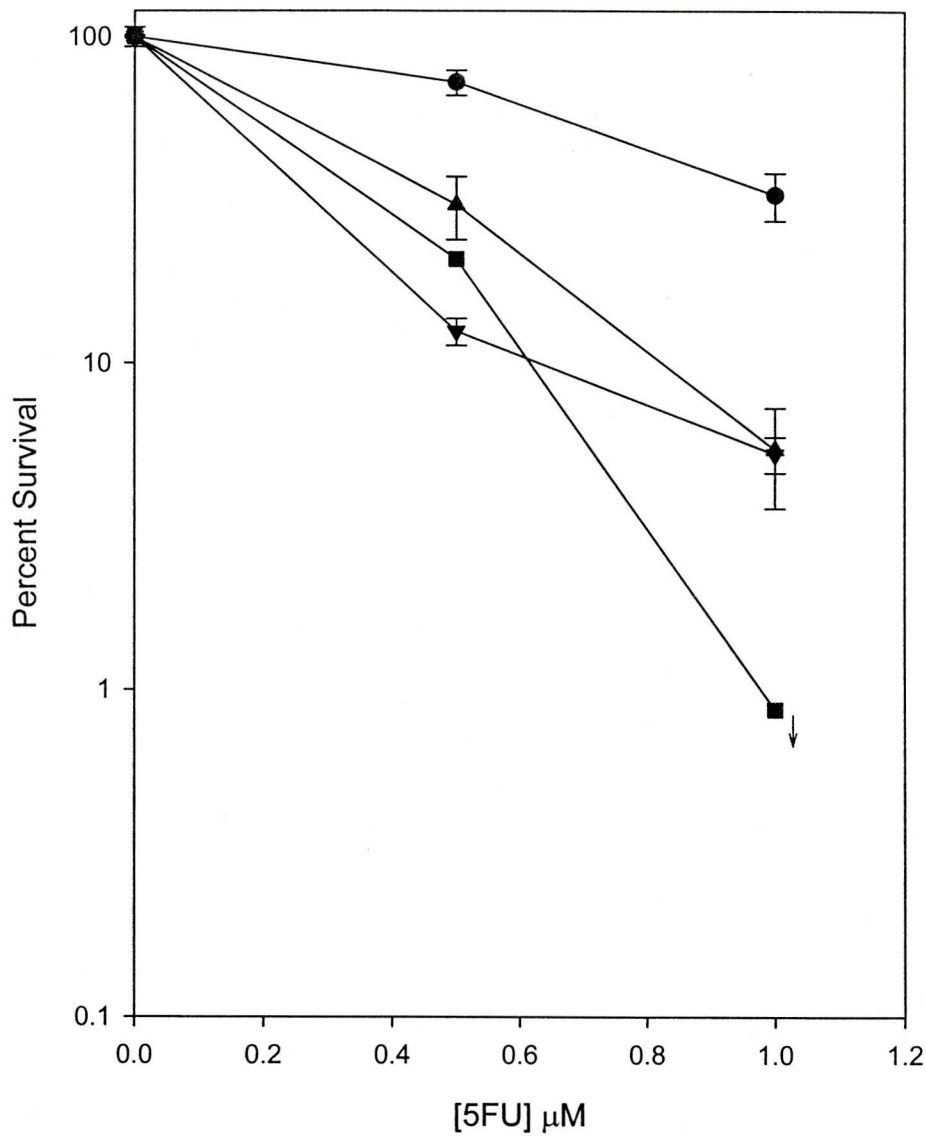
Figure 4.2. *Comparison of two different preparations of 5FC: Sigma Chemical co. and ICN Pharmaceuticals.* F10 cells were mock infected or infected with AdDL70-3 or AdCDA and exposed to 5FC obtained from Sigma Chemical Co (○, △,□) or ICN Pharmaceuticals (●, ▲,■) for 48 h. Cells were cultured for a further 3 days in drug-free media. Each datum point represents mean ± SE for a single experiment conducted in triplicate.


100
10
1
0.1
Percent Survival
0
1
2
3
4
5
6
[5FC] mM

Response of F10 cells to 5FU:

To further elucidate the response of the F10 cells to AdCDA/5FC, the dose-response to 5FU of uninfected F10 cells, MT1A2, HT29, and HT29p14 cells was determined also (Figure 4.3). After 48 hours exposure to 5FU followed by a further 4 days (F10 cells) or 5 days (MT1A2, HT29, HT29p14 cells) culture in drug-free growth media, it was shown that F10s were most resistant to 5FU. HT29 and HT29p14 cells were approximately equal in sensitivity to 5FU, while the MT1A2 cells were the most sensitive to 5FU.

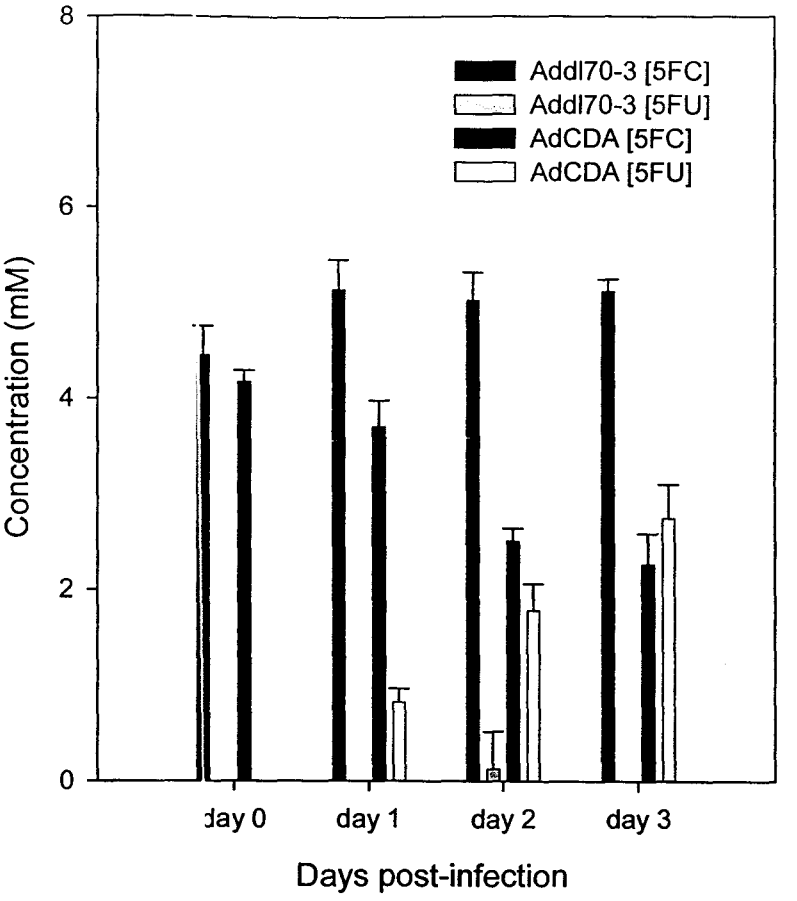
Figure 4.3. *Clonogenic survival of F10, MT1A2, HT29, HT29p14 cells exposed to 5FU.* F10 (●), MT1A2 (■), HT29 (▲), and HT29p14 (▼) cells were plated at 200 cells/well and 5FU was added 3 h post-plating. After 48 h 5FU was removed and cells were cultured in drug-free media for a further 4 days (F10) or 5 days (MT1A2, HT29, HT29p14). Each datum point represents the mean + SE for a single experiment conducted in triplicate. Downward arrow refers to a less-than value since no colonies were observed for MT1A2 cells at this dose of 5FU.


100
10
1
0.1
Percent Survival
0.0
0.2
0.4
0.6
0.8
1.0
1.2
[5FU] μM

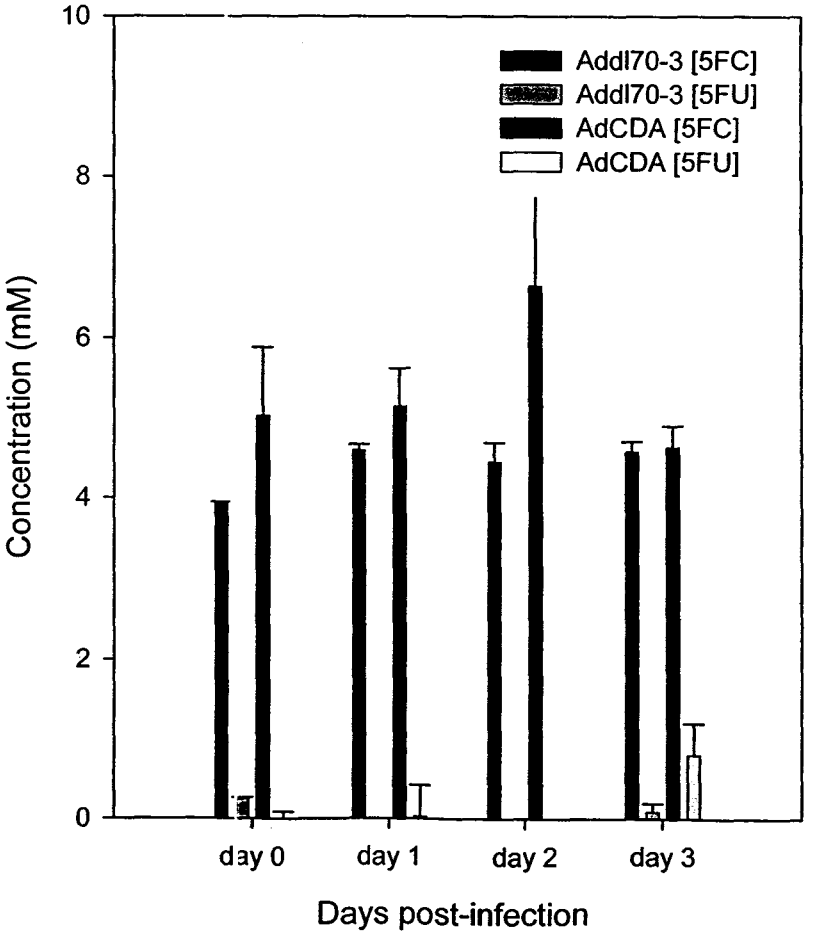
Conversion of 5FC to 5FU in AdCDA infected F10 cells:

To determine the levels of CDA expression, conversion of 5FC to 5FU was measured directly (Figure 4.4A, B). Samples of cell culture supernatants were collected at 24-h intervals, beginning immediately following the application of 5FC to F10 and MT1A2 cells infected with AdDL70-3 or AdCDA (MOI 50) and cultured in media with or without ICN 5FC. Concentrations of 5FC and 5FU were determined spectrophotometrically as described (Wallace *et al.*, 1994). By day 3 there were measurable levels of 5FU in only the media of AdCDA infected MT1A2 cells and not in that of AdDL70-3 infected cells (Figure 4.4A). By contrast, measurable levels of 5FU were not present in the media of either the AdCDA or the AdDL70-3 infected F10 cells (Figure 4.4B). This suggested that the level of CDA expression was reduced in infected F10 cells relative to MT1A2 cells.

Figure 4.4. *CDA enzymatic activity measured by spectrophotometric assay of 5FC and 5FU in culture supernatants sampled from AdCDA infected cells.* (A) MT1A2 cells were infected with AdDL70-3 and AdCDA at an MOI of 5 and (B) F10 cells were infected with AdDL70-3 or AdCDA at an MOI of 50. Cells were cultured in 0 mM or 5 mM 5FC (ICN) throughout the duration of the assay. Culture supernatant samples were obtained at approximately 24 h intervals and optical density at 255 nM and 290 nM was measured by spectrophotometer. Each datum point represents the mean $\pm$ SE of a single experiment performed in triplicate.


Addl70-3 [5FC]
Addl70-3 [5FU]
AdCDA [5FC]
AdCDA [5FU]
Concentration (mM)
0
2
4
6
8
day 0
day 1
day 2
day 3
Days post-infection

A

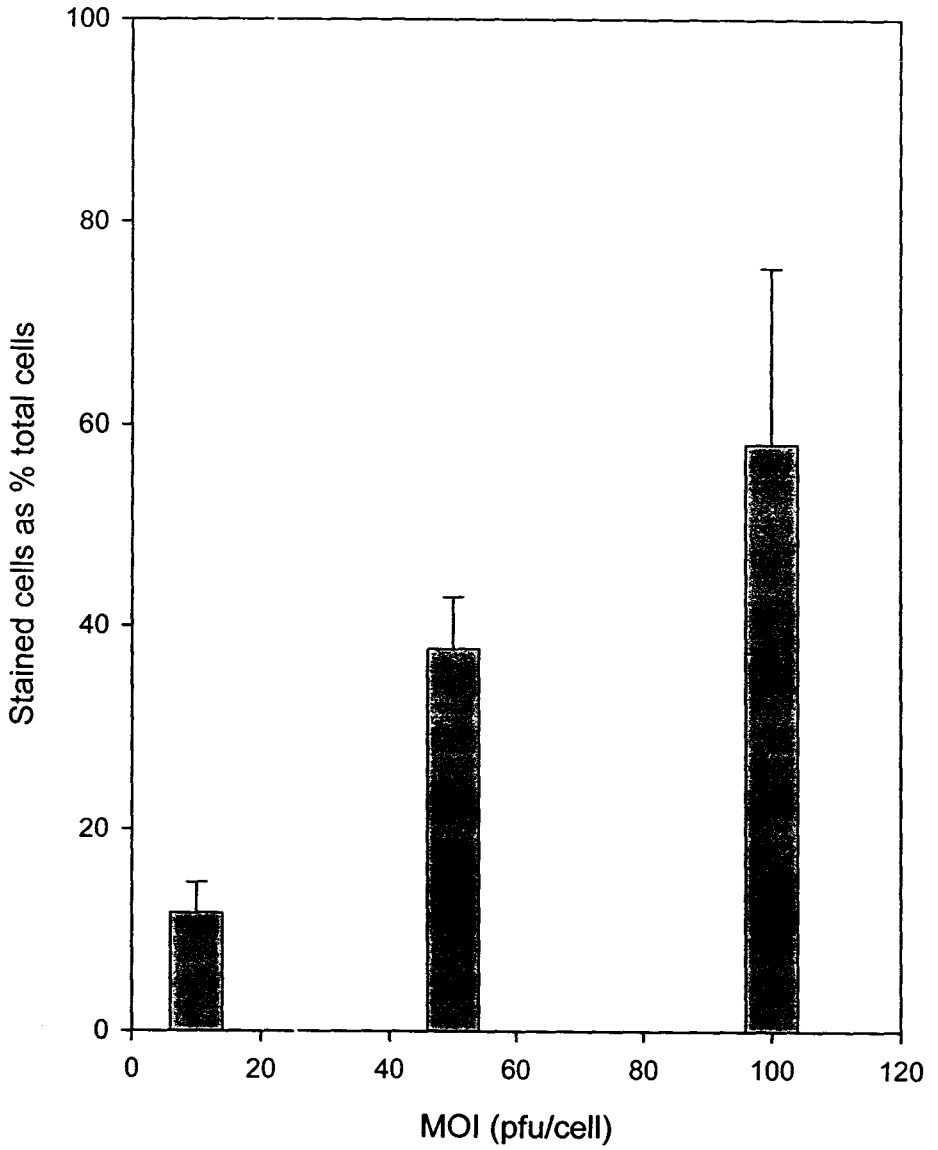

Addl70-3 [5FC]
Addl70-3 [5FU]
AdCDA [5FC]
AdCDA [5FU]
Concentration (mM)
0
2
4
6
8
10
day 0
day 1
day 2
day 3
Days post-infection

B

β-Galactosidase Staining of AdLacZ infected F10 Cells

It was known, *a priori,* that high levels of adenovirus transduction of F10 cells is difficult to achieve (M. Hitt and Y. Wan, unpublished observations). Under the same conditions as the clonogenic assay (infection in minimal medium for 1h, aspirate virus, replace in culture in fresh growth media) a β-galactosidase (β-Gal) assay was carried out to determine the levels of transduction achieved using the AdLacZ vector over a range of MOI (Figure 4.5). Adenovirus infection of F10 cells at an moi of 50, the level used in the AdCDA/5FC clonogenic assays, showed that approximately 37% of the cells stained positive for β-gal.

Figure 4.5. Xgal staining of AdLacZ infected F10 cells. F10 cells were infected with AdLacZ at an MOI of 10, 50, and 100 for 1 h as described in the Materials and Methods, and cultured for 2 days. β-galactosidase activity was assessed by Xgal staining, carried out as described in the Materials and Methods. Values graphed represent the mean $\pm$ SE of positively stained cells (stained blue) as a proportion of the total number of cells present on the haemocytometer; data representative triplicate values obtained from a single experiment.


100
80
60
40
20
0
Stained cells as % total cells
0
20
40
60
80
100
120
MOI (pfu/cell)

Attempts to reproduce the preliminary F10 experiments rendered results that were inconsistent with the preliminary data generated for F10 cells as well with the results of similar experiments carried out using MT1A2, HT29, and HT29p14 cells. The original data suggested that F10 cells infected with AdCDA exhibited a dose response to 5FC with a corresponding LD_{50} value of 15 mM in contrast to subsequent experiments that showed complete killing at a concentration of 5 mM 5FC. The discussion below attempts to address the reason for the apparent irreproducibility of AdCDA/5FC dose response curves in F10 cells.

Unpublished data (A. Schuh, 2000) indicated that variation between preparations of 5FC obtained from different manufacturers, or different lots from the same manufacturer, could be significant and ultimately affect experimental results. This suggested that contaminants in the 5FC may have been responsible for the difference in cytotoxicity observed in the two repetitions of the same experiment, since two different lots # s were used. A dose response curve comparing 5FC from Sigma and from ICN showed that it is indeed possible for different preparations of 5FC to affect cells differently. At a concentration of 5 mM, Sigma 5FC was highly toxic to both AdCDA and AdDL70-3 infected F10 cells while, at the same concentrations, 5FC from ICN was not toxic to either population of cells. However, at a dose of 25 mM 5FC there were no surviving colonies associated with either preparation.

The most probable candidate for a contaminant in preparations of 5FC was thought to be 5FU, implying that Sigma 5FC contained more contaminant 5FU than ICN 5FC. Clinical data shows that the contamination of 5FC by 5FU contributed to the toxicity experienced by patients administered 5FC as an antimicrobial chemotherapeutic and is related to the use of 5FU in the manufacture of 5FC (Vermes *et al.*, 1999). In addition, MT1A2, HT29, and HT29p14 cells infected with AdDL70-3 exhibited a slight reduction in survival at 25 mM ICN 5FC. 5FU present as a contaminant in 5FC could account for a reduction in survival occurring in the absence of CDA. The reduction in survival of F10 cells infected with either AdCDA or AdDL70 exposed to the same concentration of 5FC suggested that F10 cells may be hypersensitive to 5FU. To address whether F10 cells are relatively hypersensitive to 5FU the dose-response to 5FU of all 4 cell lines was determined. This assay demonstrated, however, that F10 cells

are less sensitive to 5FU than MT1A2, HT29, and HT29p14 cells. Thus the contaminant in 5FC preparations used here is unlikely to be 5FU.

No AdCDA-dependent killing in the presence of 5FC was observed in infected F10 cell cultures. The lack of CDA-dependent killing could be attributable to an insensitivity to 5FU, as demonstrated in Figure 6, or a lack of CDA expression in the infected cultures, among other possibilities. To investigate whether or not 5FC was being converted to 5FU by infected F10 and MT1A2 cells, a spectrophotometric assay of culture supernatants was performed. In the AdCDA infected MT1A2 cell cultures, 5FC was converted to 5FU beginning on day 1 of the assay and continuing to day 3. Measurable amounts of 5FU were detected only in the presence of AdCDA. In contrast, 5FU was only just detectable in AdCDA infected F10 cell cultures by day 3 of the experiment. This is significant because the conditions of the clonogenic assay are such that cells are exposed to 5FC for only 2 days. Thus, minimal 5FU was generated by infected F10 cells prior to prodrug withdrawal.

One possible mechanism that would affect conversion of 5FC to 5FU is the extent to which F10 cells were transduced by the AdCDA virus. Transduction efficiency was determined by Xgal staining for β-gal expression 48 hours after infecting F10 cells with AdLacz at an MOI of 50. Under these conditions, 40% of the F10 cells stained blue. Interpretation of these results is limited because the β-gal assay employed only allows differentiation between cells that express and cells that do not express the reporter gene rather than ascertaining the levels of transgene expression. The latter could be accomplished using a standard β-gal assay, but using ONPG as a substrate rather than Xgal, as described in Francis, 2000b. The level of β-gal expressed in F10 cells infected with Adlacz then cultured in the presence or absence of 5FC might allow one to determine whether some contaminant in the 5FC preparation interferes with global gene expression. Alternatively, the contaminant 5FC might in some way specifically block CDA expression or activity in F10 cells.

This examination reveals an important considerations for the assessment of treatments where a non-toxic substance is enzymatically converted to an active, cytotoxic substance. Namely, that it is possible for some contaminant or additive in the preparation of the compound to be toxic to one or more cell lines evaluated. This would manifest as a false-positive result. It is thus important to include the

appropriate controls in evaluations of the cytotoxicity of an enzyme-prodrug treatment to accommodate evaluation of the cytotoxic effect of the prodrug in the absence of the appropriate converting enzyme.

CHAPTER 5

REFERENCES

Amrein, P. C. & Weitzman, S. A. (1985). Treatment of squamous-cell carcinoma of the head and neck with cisplatin and 5-fluorouracil. *J Clin Oncol* **3**, 1632-9.

Andersen, L., Kilstrup, M. & Neuhard, J. (1989). Pyrimidine, purine and nitrogen control of cytosine deaminase synthesis in Escherichia coli K 12. Involvement of the glnLG and purR genes in the regulation of codA expression. *Arch Microbiol* **152**, 115-8.

Anderson, A. C., O'Neil, R. H., DeLano, W. L. & Stroud, R. M. (1999). The structural mechanism for half-the-sites reactivity in an enzyme, thymidylate synthase, involves a relay of changes between subunits. *Biochemistry* **38**, 13829-36.

Arcangeli, G., Angelini, F., Tersigni, R., D'Aprile, M., Micheli, A., Veltri, E., Ambrogi, C., Alessandroni, L., Giovinazzo, G. & et al. (1995). A pilot study of concomitant protracted venous infusion 5-fluorouracil and hyperfractionated radiotherapy in rectal tumors. *Am J Clin Oncol* **18**, 369-75.

Aschele, C., Guglielmi, A., Frassineti, G. L., Milandri, C., Amadori, D., Labianca, R., Vinci, M., Tixi, L., Caroti, C., Ciferri, E., Verdi, E., Rosso, R. & Sobrero, A. (1998). Schedule-selective biochemical modulation of 5-fluorouracil in advanced colorectal cancer: a multicentric phase II study. *Br J Cancer* **77**, 341-6.

Austin, E. A. & Huber, B. E. (1993). A first step in the development of gene therapy for colorectal carcinoma: cloning, sequencing, and expression of Escherichia coli cytosine deaminase. *Mol Pharmacol* **43**, 380-7.

Bett, A. J., Haddara, W., Prevec, L. & Graham, F. L. (1994). An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc Natl Acad Sci U S A* **91**, 8802-6.

Bevington, P. (1969). Data reduction and eorror analysis for the physical sciences. New York: McGraw-Hill).

Blackstock, A. W., Kwock, L., Branch, C., Zeman, E. M. & Tepper, J. E. (1996). Tumor retention of 5-fluorouracil following irradiation observed using 19F nuclear magnetic resonance spectroscopy. *Int J Radiat Oncol Biol Phys* **36**, 641-8.

Block, A., Freund, C. T., Chen, S. H., Nguyen, K. P., Finegold, M., Windler, E. & Woo, S. L. (2000). Gene therapy of metastatic colon carcinoma: regression of multiple hepatic metastases by adenoviral expression of bacterial cytosine deaminase. *Cancer Gene Ther* **7**, 438-45.

Brabec, V., Sip, M. & Leng, M. (1993). DNA conformational change produced by the site-specific interstrand cross-link of trans-diamminedichloroplatinum(II). *Biochemistry* **32**, 11676-81.

Bramson, J. L., Graham, F. L. & Gauldie, J. (1995). The use of adenoviral vectors for gene therapy and gene transfer in vivo. *Curr Opin Biotechnol* **6**, 590-5.

Byfield, J. E. (1989). 5-Fluorouracil radiation sensitization--a brief review. *Invest New Drugs* **7**, 111-6.

Calabro-Jones, P. M., Byfield, J. E., Ward, J. F. & Sharp, T. R. (1982). Time-dose relationships for 5-fluorouracil cytotoxicity against human epithelial cancer cells in vitro. *Cancer Res* **42**, 4413-20.

Caney, C., Bulmer, J. T., Singh, G., Lukka, H. & Rainbow, A. J. (1999). Pre-exposure of human squamous carcinoma cells to low-doses of gamma-rays leads to an increased resistance to subsequent low-dose cisplatin treatment. *Int J Radiat Biol* **75**, 963-72.

Cao, X., Ju, D. W., Tao, Q., Wang, J., Wan, T., Wang, B. M., Zhang, W. & Hamada, H. (1998). Adenovirus-mediated GM-CSF gene and cytosine deaminase gene transfer followed by 5-fluorocytosine administration elicit more potent antitumor response in tumor-bearing mice. *Gene Ther* **5**, 1130-6.

Cavrini, V., Bonazzi, D. & Di Pietra, A. M. (1991). Analysis of flucytosine dosage forms by derivative UV spectroscopy and liquid chromatography. *J Pharm Biomed Anal* **9**, 401-7.

Christ, M., Lusky, M., Stoeckel, F., Dreyer, D., Dieterle, A., Michou, A. I., Pavirani, A. & Mehtali, M. (1997). Gene therapy with recombinant adenovirus vectors: evaluation of the host immune response. *Immunol Lett* **57**, 19-25.

Chu, G. (1994). Cellular responses to cisplatin. The roles of DNA binding-proteins and DNA repair. *AJ. Biol. Chem.* **969**, 787-790.

Consalvo, M., Mullen, C. A., Modesti, A., Musiani, P., Allione, A., Cavallo, F., Giovarelli, M. & Forni, G. (1995). 5-Fluorocytosine-induced eradication of murine adenocarcinomas engineered to express the cytosine deaminase suicide gene requires host immune competence and leaves an efficient memory. *J Immunol* **154**, 5302-12.

Cotran, R., Kumar V., & Collins T. (1999). Pathologic Basis of Disease. (Philedelphia: W.B. Saunders Company).

Danenberg, P. V. (1977). Thymidylate synthetase - a target enzyme in cancer chemotherapy. *Biochim Biophys Acta* **473**, 73-92.

Danenberg, P. V. & Lockshin, A. (1982). Tight-binding complexes of thymidylate synthetase, folate analogs and deoxyribonucleotides. *Adv Enzyme Regul* **20**, 99-110.

Datta, R., Rubin, E., Sukhatme, V., Qureshi, S., Hallahan, D., Weichselbaum, R. R. & Kufe, D. W. (1992). Ionizing radiation activates transcription of the EGR1 gene via CArG elements. *Proc Natl Acad Sci U S A* **89**, 10149-53.

Decker, D. A., Drelichman, A., Jacobs, J., Hoschner, J., Kinzie, J., Loh, J. J., Weaver, A. & Al-Sarraf, M. (1983). Adjuvant chemotherapy with cis-diamminodichloroplatinum II and 120-hour infusion 5-fluorouracil in Stage III and IV squamous cell carcinoma of the head and neck. *Cancer* **51**, 1353-5.

Diasio, R. B., Bennett, J. E. & Myers, C. E. (1978). Mode of action of 5-fluorocytosine. *Biochem Pharmacol* **27**, 703-7.

Dilber, M. S. & Gahrton, G. (2001). Suicide gene therapy: possible applications in haematopoietic disorders. *J Intern Med* **249**, 359-67.

Dilber, M. S. & Smith, C. I. (1997). Suicide genes and bystander killing: local and distant effects. *Gene Ther* **4**, 273-4.

Dolnick, B. J. & Pink, J. J. (1985). Effects of 5-fluorouracil on dihydrofolate reductase and dihydrofolate reductase mRNA from methotrexate-resistant KB cells. *J Biol Chem* **260**, 3006-14.

Dong, Y., Wen, P., Manome, Y., Parr, M., Hirshowitz, A., Chen, L., Hirschowitz, E. A., Crystal, R., Weichselbaum, R., Kufe, D. W. & Fine, H. A. (1996). In vivo replication-deficient adenovirus vector-mediated transduction of the cytosine deaminase gene sensitizes glioma cells to 5-fluorocytosine. *Hum Gene Ther* **7**, 713-20.

Dougherty, T. J., Gomer, C. J., Henderson, B. W., Jori, G., Kessel, D., Korbelik, M., Moan, J. & Peng, Q. (1998). Photodynamic therapy. *J Natl Cancer Inst* **90**, 889-905.

Esaki, T., Nakano, S., Tatsumoto, T., Kuroki-Migita, M., Mitsugi, K., Nakamura, M. & Niho, Y. (1992). Inhibition by 5-fluorouracil of cis-diamminedichloroplatinum(II)-induced DNA interstrand cross-link removal in a HST-1 human squamous carcinoma cell line. *Cancer Res* **52**, 6501-6.

Fidler, I. J. & Kripke, M. L. (1977). Metastasis results from preexisting variant cells within a malignant tumor. *Science* **197**, 893-5.

Fogh, J., Fogh, J. M. & Orfeo, T. (1977). One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst* **59**, 221-6.

Frei, E., 3rd, Elias, A., Wheeler, C., Richardson, P. & Hryniuk, W. (1998). The relationship between high-dose treatment and combination chemotherapy: the concept of summation dose intensity. *Clin Cancer Res* **4**, 2027-37.

Gately, D. P. & Howell, S. B. (1993). Cellular accumulation of the anticancer agent cisplatin: a review. *Br J Cancer* **67**, 1171-6.

Glazer, R. I. & Peale, A. L. (1979). The effect of 5-fluorouracil on the synthesis of nuclear RNA in L1210 cells in vitro. *Mol Pharmacol* **16**, 270-7.

Gnant, M. F., Puhlmann, M., Alexander, H. R., Jr. & Bartlett, D. L. (1999). Systemic administration of a recombinant vaccinia virus expressing the cytosine deaminase gene and subsequent treatment with 5-fluorocytosine leads to tumor-specific gene expression and prolongation of survival in mice. *Cancer Res* **59**, 3396-403.

Gonzalez, V. M., Fuertes, M. A., Alonso, C. & Perez, J. M. (2001). Is cisplatin-induced cell death always produced by apoptosis? *Mol Pharmacol* **59**, 657-63.

Graham, F. L., Smiley, J., Russell, W. C. & Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* **36**, 59-74.

Grem, J. L. (2000). 5-Fluorouracil: forty-plus and still ticking. A review of its preclinical and clinical development. *Invest New Drugs* **18**, 299-313.

Guy, C. T., Cardiff, R. D. & Muller, W. J. (1992). Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol Cell Biol* **12**, 954-61.

Hamstra, D. A., Rice, D. J., Pu, A., Oyedijo, D., Ross, B. D. & Rehemtulla, A. (1999). Combined radiation and enzyme/prodrug treatment for head and neck cancer in an orthotopic animal model. *Radiat Res* **152**, 499-507.

Hanna, N. N., Mauceri, H. J., Wayne, J. D., Hallahan, D. E., Kufe, D. W. & Weichselbaum, R. R. (1997). Virally directed cytosine deaminase/5-fluorocytosine gene therapy enhances radiation response in human cancer xenografts. *Cancer Res* **57**, 4205-9.

Heidelberger, C., Danenberg, P. V. & Moran, R. G. (1983). Fluorinated pyrimidines and their nucleosides. *Adv Enzymol Relat Areas Mol Biol* **54**, 58-119.

Hierholzer, J. C., Wigand, R., Anderson, L. J., Adrian, T. & Gold, J. W. (1988). Adenoviruses from patients with AIDS: a plethora of serotypes and a description of five new serotypes of subgenus D (types 43-47). *J Infect Dis* **158**, 804-13.

Hirschowitz, E. A., Ohwada, A., Pascal, W. R., Russi, T. J. & Crystal, R. G. (1995). In vivo adenovirus-mediated gene transfer of the Escherichia coli cytosine deaminase gene to human colon carcinoma-derived tumors induces chemosensitivity to 5-fluorocytosine. *Hum Gene Ther* **6**, 1055-63.

Hitt, M. M., Addison, C. L. & Graham, F. L. (1997). Human adenovirus vectors for gene transfer into mammalian cells. *Adv Pharmacol* **40**, 137-206.

Hitt, M. M. & Graham, F. L. (2000). Adenovirus vectors for human gene therapy. *Adv Virus Res* **55**, 479-505.

Hitt, M. M., Parks, R. J. & Graham, F. L. (1999). Structure and genetic organization of adenovirus vectors. In *The Development of Human Gene Therapy*, pp. 61-86. Edited by T. Friedmann. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Holder, J. W., Elmore, E. & Barrett, J. C. (1993). Gap junction function and cancer. *Cancer Res* **53**, 3475-85.

Huber, B. E., Austin, E. A., Good, S. S., Knick, V. C., Tibbels, S. & Richards, C. A. (1993). In vivo antitumor activity of 5-fluorocytosine on human colorectal carcinoma cells genetically modified to express cytosine deaminase. *Cancer Res* **53**, 4619-26.

Huber, B. E., Austin, E. A., Richards, C. A., Davis, S. T. & Good, S. S. (1994). Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. *Proc Natl Acad Sci U S A* **91**, 8302-6.

Ingraham, H. A., Tseng, B. Y. & Goulian, M. (1980). Mechanism for exclusion of 5-fluorouracil from DNA. *Cancer Res* **40**, 998-1001.

Jin, Y., Heck, D. E., DeGeorge, G., Tian, Y. & Laskin, J. D. (1996). 5-Fluorouracil suppresses nitric oxide biosynthesis in colon carcinoma cells. *Cancer Res* **56**, 1978-82.

Johnston, P. G., Geoffrey, F., Drake, J., Voeller, D., Grem, J. L. & Allegra, C. J. (1996). The cellular interaction of 5-fluorouracil and cisplatin in a human colon carcinoma cell line. *Eur J Cancer* **32A**, 2148-54.

Kemeny, N., Israel, K., Niedzwiecki, D., Chapman, D., Botet, J., Minsky, B., Vinciguerra, V., Rosenbluth, R., Bosselli, B., Cochran, C. & et al. (1990). Randomized study of continuous infusion fluorouracil versus fluorouracil plus cisplatin in patients with metastatic colorectal cancer. *J Clin Oncol* **8**, 313-8.

Khil, M. S., Kim, J. H., Mullen, C. A., Kim, S. H. & Freytag, S. O. (1996). Radiosensitization by 5-fluorocytosine of human colorectal carcinoma cells in culture transduced with cytosine deaminase gene. *Clin Cancer Res* **2**, 53-7.

Kievit, E., Bershad, E., Ng, E., Sethna, P., Dev, I., Lawrence, T. S. & Rehemtulla, A. (1999). Superiority of yeast over bacterial cytosine deaminase for enzyme/prodrug gene therapy in colon cancer xenografts. *Cancer Res* **59**, 1417-21.

Kilstrup, M., Meng, L. M., Neuhard, J. & Nygaard, P. (1989). Genetic evidence for a repressor of synthesis of cytosine deaminase and purine biosynthesis enzymes in Escherichia coli. *J Bacteriol* **171**, 2124-7.

Kirkwood F., Lotze M., & Yasko J. (1996). Current Cancer Therapeutics. (Philedelphia: Current Medicine).

Koyama, F., Sawada, H., Fujii, H., Hirao, T., Ueno, M., Hamada, H. & Nakano, H. (2000). Enzyme/prodrug gene therapy for human colon cancer cells using adenovirus-mediated

transfer of the Escherichia coli cytosine deaminase gene driven by a CAG promoter associated with 5-fluorocytosine administration. *J Exp Clin Cancer Res* **19**, 75-80.

Kurian, K. M., Watson, C. J. & Wyllie, A. H. (2000). Retroviral vectors. *Mol Pathol* **53**, 173-6.

Kuriyama, S., Kikukawa, M., Masui, K., Okuda, H., Nakatani, T., Sakamoto, T., Yoshiji, H., Fukui, H., Ikenaka, K., Mullen, C. A. & Tsujii, T. (1999). Cytosine deaminase/5-fluorocytosine gene therapy can induce efficient anti-tumor effects and protective immunity in immunocompetent mice but not in athymic nude mice. *Int J Cancer* **81**, 592-7.

Kuriyama, S., Masui, K., Sakamoto, T., Nakatani, T., Kikukawa, M., Tsujinoue, H., Mitoro, A., Yamazaki, M., Yoshiji, H., Fukui, H., Ikenaka, K., Mullen, C. A. & Tsujii, T. (1998). Bystander effect caused by cytosine deaminase gene and 5-fluorocytosine in vitro is substantially mediated by generated 5-fluorouracil. *Anticancer Res* **18**, 3399-406.

Lacave, A. J., Baron, F. J., Anton, L. M., Estrada, E., De Sande, L. M., Palacio, I., Esteban, E., Gracia, J. M., Buesa, J. M., Fernandez, O. A. & et al. (1991). Combination chemotherapy with cisplatin and 5-fluorouracil 5-day infusion in the therapy of advanced gastric cancer: a phase II trial. *Ann Oncol* **2**, 751-4.

Lattime E., & Gerson S. (1999). Gene Therapy of Cancer. (San Diego: Academic Press).

Lawrence, T. S., Davis, M. A. & Maybaum, J. (1994). Dependence of 5-fluorouracil-mediated radiosensitization on DNA-directed effects. *Int J Radiat Oncol Biol Phys* **29**, 519-23.

Levard, H., Pouliquen, X., Hay, J. M., Fingerhut, A., Langlois-Zantain, O., Huguier, M., Lozach, P. & Testart, J. (1998). 5-Fluorouracil and cisplatin as palliative treatment of advanced oesophageal squamous cell carcinoma. A multicentre randomised controlled trial. The French Associations for Surgical Research. *Eur J Surg* **164**, 849-57.

Li, Z., Shanmugam, N., Katayose, D., Huber, B., Srivastava, S., Cowan, K. & Seth, P. (1997). Enzyme/prodrug gene therapy approach for breast cancer using a recombinant adenovirus expressing Escherichia coli cytosine deaminase. *Cancer Gene Ther* **4**, 113-7.

Loehrer, P. J., Sr., Einhorn, L. H., Williams, S. D., Hui, S. L., Estes, N. C. & Pennington, K. (1985). Cisplatin plus 5-FU for the treatment of adenocarcinoma of the colon. *Cancer Treat Rep* **69**, 1359-63.

Lodish, H., Baltimore, D., Berk, A., Zipursky, S.L., Matsudaira, P., & Darnell, J. (1995). Molecular Cell Biology. New York, NY: W. H. Freeman and Company, New York.

Macdonald, J. S. (1999). Toxicity of 5-fluorouracil. *Oncology (Huntingt)* **13**, 33-4.

Mittal, S. K., McDermott, M. R., Johnson, D. C., Prevec, L. & Graham, F. L. (1993). Monitoring foreign gene expression by a human adenovirus-based vector using the firefly luciferase gene as a reporter. *Virus Res* **28**, 67-90.

Moorehead, R. A., Armstrong, S. G., Wilson, B. C. & Singh, G. (1994). Cross-resistance to cisplatin in cells resistant to photofrin-mediated photodynamic therapy. *Cancer Res* **54**, 2556-9.

Morere, J. F., Duran, A., Tcherakian, F., Boaziz, C., Valeyre, D., Battesti, J. P., Breu, J. L. & Israel, L. (1994). Cisplatin-5-fluorouracil in small cell lung cancer. A phase II study in 109 patients. *Lung Cancer* **11**, 275-81.

Morris, J.C. (1999). Suicide Gene Therapy. In *The Development of Human Gene Therapy*, pp. 477-526. Edited by T. Friedmann. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Mullen, C. A. (1994). Metabolic suicide genes in gene therapy. *Pharmacol Ther* **63**, 199-207.

Mullen, C. A., Coale, M. M., Lowe, R. & Blaese, R. M. (1994). Tumors expressing the cytosine deaminase suicide gene can be eliminated in vivo with 5-fluorocytosine and induce protective immunity to wild type tumor. *Cancer Res* **54**, 1503-6.

Mullen, C. A., Kilstrup, M. & Blaese, R. M. (1992). Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system. *Proc Natl Acad Sci U S A* **89**, 33-7.

Mullen, C. A., Petropoulos, D. & Lowe, R. M. (1996). Treatment of microscopic pulmonary metastases with recombinant autologous tumor vaccine expressing interleukin 6 and Escherichia coli cytosine deaminase suicide genes. *Cancer Res* **56**, 1361-6.

Myers, C. E., Diasio, R., Eliot, H. M. & Chabner, B. A. (1976). Pharmacokinetics of the fluoropyrimidines: implications for their clinical use. *Cancer Treat Rev* **3**, 175-83.

Myers, C. E., Young, R. C. & Chabner, B. A. (1975). Biochemical determinants of 5-fluorouracil response in vivo. The role of deoxyuridylate pool expansion. *J Clin Invest* **56**, 1231-8.

Nanni, P., De Giovanni, C., Nicoletti, G., Landuzzi, L., Rossi, I., Frabetti, F., Giovarelli, M., Forni, G., Cavallo, F., Di Carlo, E., Musiani, P. & Lollini, P. L. (1998). The immune response elicited by mammary adenocarcinoma cells transduced with interferon-gamma and cytosine deaminase genes cures lung metastases by parental cells. *Hum Gene Ther* **9**, 217-24.

Ohwada, A., Hirschowitz, E. A. & Crystal, R. G. (1996). Regional delivery of an adenovirus vector containing the Escherichia coli cytosine deaminase gene to provide local activation of 5-fluorocytosine to suppress the growth of colon carcinoma metastatic to liver. *Hum Gene Ther* **7**, 1567-76.

Palmeri, S., Trave, F., Russello, O. & Rustum, Y. M. (1989). The role of drug sequence in therapeutic selectivity of the combination of 5-fluorouracil and cis-platin. *Sel Cancer Ther* **5**, 169-77.

Perez, R. P. (1998). Cellular and molecular determinants of cisplatin resistance. *Eur J Cancer* **34**, 1535-42.

Peters, G. J., Laurensse, E., Leyva, A., Lankelma, J. & Pinedo, H. M. (1986). Sensitivity of human, murine, and rat cells to 5-fluorouracil and 5'-deoxy-5-fluorouridine in relation to drug-metabolizing enzymes. *Cancer Res* **46**, 20-8.

Peters, G. J., van der Wilt, C. L., van Moorsel, C. J., Kroep, J. R., Bergman, A. M. & Ackland, S. P. (2000a). Basis for effective combination cancer chemotherapy with antimetabolites. *Pharmacol Ther* **87**, 227-53.

Peters, G. J., van Triest, B., Backus, H. H., Kuiper, C. M., van der Wilt, C. L. & Pinedo, H. M. (2000b). Molecular downstream events and induction of thymidylate synthase in mutant and wild-type p53 colon cancer cell lines after treatment with 5-fluorouracil and the thymidylate synthase inhibitor raltitrexed. *Eur J Cancer* **36**, 916-24.

Pierrefite-Carle, V., Baque, P., Gavelli, A., Mala, M., Chazal, M., Gugenheim, J., Bourgeon, A., Milano, G., Staccini, P. & Rossi, B. (1999). Cytosine deaminase/5-fluorocytosine-based vaccination against liver tumors: evidence of distant bystander effect. *J Natl Cancer Inst* **91**, 2014-9.

Pinedo, H. M. & Peters, G. F. (1988). Fluorouracil: biochemistry and pharmacology. *J Clin Oncol* **6**, 1653-64.

Putzer, B. M., Hitt, M., Muller, W. J., Emtage, P., Gauldie, J. & Graham, F. L. (1997). Interleukin 12 and B7-1 costimulatory molecule expressed by an adenovirus vector act synergistically to facilitate tumor regression. *Proc Natl Acad Sci U S A* **94**, 10889-94.

Rochlitz, C. F. (2001). Gene therapy of cancer. *Schweiz Med Wochenschr* **131**, 4-9.

Roobol, C., Sips, H. C., Theunissen, J., Atassi, G. & Bernheim, J. L. (1984). In vitro assessment of cytotoxic agents in murine cancers: comparison between antiproliferative and antimetabolic assays. *J Natl Cancer Inst* **72**, 661-6.

Rooney, M., Kish, J., Jacobs, J., Kinzie, J., Weaver, A., Crissman, J. & Al-Sarraf, M. (1985). Improved complete response rate and survival in advanced head and neck cancer after three-course induction therapy with 120-hour 5-FU infusion and cisplatin. *Cancer* **55**, 1123-8.

Rosenberg, B. (1985). Fundamental studies with cisplatin. *Cancer* **55**, 2303-16.

Rowley, S., Lindauer, M., Gebert, J. F., Haberkorn, U., Oberdorfer, F., Moebius, U., Herfarth, C. & Schackert, H. K. (1996). Cytosine deaminase gene as a potential tool for the genetic therapy of colorectal cancer. *J Surg Oncol* **61**, 42-8.

Scanlon, K. J. & Kashani-Sabet, M. (1988). Elevated expression of thymidylate synthase cycle genes in cisplatin-resistant human ovarian carcinoma A2780 cells. *Proc Natl Acad Sci U S A* **85**, 650-3.

Scanlon, K. J., Lu, Y., Kashani-Sabet, M., Ma, J. & Newman, E. (1988). Mechanisms for cisplatin-FUra synergism and cisplatin resistance in human ovarian carcinoma cells both in vitro and in vivo. *Adv Exp Med Biol* **244**, 127-35.

Scanlon, K. J., Newman, E. M., Lu, Y. & Priest, D. G. (1986). Biochemical basis for cisplatin and 5-fluorouracil synergism in human ovarian carcinoma cells. *Proc Natl Acad Sci U S A* **83**, 8923-5.

Schmoll, H. J., Buchele, T., Grothey, A. & Dempke, W. (1999). Where do we stand with 5-fluorouracil? *Semin Oncol* **26**, 589-605.

Schnipper, L. (1986). Clinical implications of tumor-cell heterogeneity. *N Engl J Med* **314**, 1423-31.

Shirakawa, T., Gardner, T. A., Ko, S. C., Bander, N., Woo, S., Gotoh, A., Kamidono, S., Chung, L. W. & Kao, C. (1999). Cytotoxicity of adenoviral-mediated cytosine deaminase plus 5-

fluorocytosine gene therapy is superior to thymidine kinase plus acyclovir in a human renal cell carcinoma model. *J Urol* **162**, 949-54.

Shirasaka, T., Shimamoto, Y., Ohshimo, H., Saito, H. & Fukushima, M. (1993). Metabolic basis of the synergistic antitumor activities of 5-fluorouracil and cisplatin in rodent tumor models in vivo. *Cancer Chemother Pharmacol* **32**, 167-72.

Shrivastav, S., Bonar, R. A., Stone, K. R. & Paulson, D. F. (1980). An in vitro assay procedure to test chemotherapeutic drugs on cells from human solid tumors. *Cancer Res* **40**, 4438-42.

Singh, G., Espiritu, M., Shen, X. Y., Hanlon, J. G. & Rainbow, A. J. (2001). In vitro induction of PDT resistance in HT29, HT1376 and SK-N-MC cells by various photosensitizers. *Photochem Photobiol* **73**, 651-6.

Springer, C. J. & Niculescu-Duvaz, I. (2000). Prodrug-activating systems in suicide gene therapy. *J Clin Invest* **105**, 1 61-7.

Stupp, R. & Vokes, E. E. (1995). 5-fluorouracil plus radiation for head and neck cancer. *J Infus Chemother* **5**, 55-60.

Szary, J., Missol, E., Tarnawski, R. & Szala, S. (1997). Selective augmentation of radiation effects by 5-fluorocytosine on murine B16(F10) melanoma cells transfected with cytosine deaminase gene. *Cancer Gene Ther* **4**, 269-72.

Takahara, P. M., Rosenzweig, A. C., Frederick, C. A. & Lippard, S. J. (1995). Crystal structure of double-stranded DNA containing the major adduct of the anticancer drug cisplatin. *Nature* **377**, 649-52.

Vermes, A., Guchelaar, H. J. & Dankert, J. (2000). Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions. *J Antimicrob Chemother* **46**, 171-9.

Vermes, A., van der Sijs, H. & Guchelaar, H. J. (1999). An accelerated stability study of 5-flucytosine in intravenous solution. *Pharm World Sci* **21**, 35-9.

Wallace, P. M., MacMaster, J. F., Smith, V. F., Kerr, D. E., Senter, P. D. & Cosand, W. L. (1994). Intratumoral generation of 5-fluorouracil mediated by an antibody-cytosine deaminase conjugate in combination with 5-fluorocytosine. *Cancer Res* **54**, 2719-23.

Washtien, W. L. (1984). Comparison of 5-fluorouracil metabolism in two human gastrointestinal tumor cell lines. *Cancer Res* **44**, 909-14.

Weckbecker, G. (1991). Biochemical pharmacology and analysis of fluoropyrimidines alone and in combination with modulators. *Pharmacol Ther* **50**, 367-424.

Wilkinson, D. S. & Crumley, J. (1977). Metabolism of 5-fluorouracil in sensitive and resistant Novikoff hepatoma cells. *J Biol Chem* **252**, 1051-6.

Wilkinson, D. S. & Pitot, H. C. (1973). Inhibition of ribosomal ribonucleic acid maturation in Novikoff hepatoma cells by 5-fluorouracil and 5-fluorouridine. *J Biol Chem* **248**, 63-8.

Williamson, S. K., Tangen, C. M., Maddox, A. M., Spiridonidis, C. H. & Macdonald, J. S. (1995). Phase II evaluation of low-dose continuous 5-fluorouracil and weekly cisplatin in advanced adenocarcinoma of the stomach. A Southwest Oncology Group study. *Am J Clin Oncol* **18**, 484-7.

Wolff, G., Korner, I. J., Schumacher, A., Arnold, W., Dorken, B. & Mapara, M. Y. (1998). Ex vivo breast cancer cell purging by adenovirus-mediated cytosine deaminase gene transfer and short-term incubation with 5-fluorocytosine completely prevents tumor growth after transplantation. *Hum Gene Ther* **9**, 2277-84.

Yang, Y., Nunes, F. A., Berencsi, K., Furth, E. E., Gonczol, E. & Wilson, J. M. (1994). Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc Natl Acad Sci U S A* **91**, 4407-11.

Zhang, S. N., Yuan, S. Z., Zhu, Z. H., Wen, Z. F., Huang, Z. Q. & Zeng, Z. Y. (2000). Apoptosis induced by 5-flucytosine in human pancreatic cancer cells genetically modified to express cytosine deaminase. *Acta Pharmacol Sin* **21**, 655-9.

Zlatanova, J., Yaneva, J., & Leuba, S. H. (1998). Proteins that specifically recognize cisplatin-damaged DNA: a clue to anticancer activity of cisplatin. *AFASDB J.* **12**, 791-799.