

**Viral HSV1-TK Gene, Radiolabeled FIAU, and
Ganciclovir: Combined Gene Targeted Radiotherapy
and Suicide Gene Therapy for Prostate Cancer**

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**A thesis submitted to the University of Glasgow for the Degree of
Doctor of Medicine**

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Molecular Pathology, CRUK Beatson Laboratories
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February 2008

To my parents

Acknowledgement

First of all, I would like to acknowledge my sincere debt to Professor David Kirk, my clinical supervisor, for providing me with this outstanding opportunity to undertake this rather challenging and inspiring project for which he provided the financial support from the Departmental Urological Cancer Research Fund, Gartnavel General Hospital.

It was a real honour and privilege to be supervised by two pioneers in targeted therapy, Drs Robert Mairs and Marie Boyd for whom I am grateful to their unrestricted advice, support and encouragement.

I am really grateful to Lesley Wilson for her valuable assistance with the tissue culture, clonogenic assays and the radionuclide uptake studies. Many thanks must go to Ann Livingston and Dr Anthony McCluskey, firstly, for introducing me to the strange environment of the laboratory and secondly, for the useful discussion of my experiments and results.

I must also thank Drs Sally Pimlott and Jonathan Owens for their great efforts to keep a well-scheduled supply of the radionuclide FIAU. I would also like to thank my educational supervisors, Mr Michael Aitchison and Mr Michael Fraser for their patience with me in taking my research related work to their operating theatres and clinics trying to spare extra time.

I have been gifted with a great wife, Huda. This thesis would not have seen the day light without her continuous support, prayer, and encouragement. Huda has also gifted me with two fantastic sons, Moaad and Farouq, whom did not allow me to write a mere sentence of my thesis at home but they were a very effective remedy for the research-related-stress.

Declaration

I hereby declare that all experiments, data collection, and statistical analysis described in this thesis were conducted by me unless mentioned otherwise in the text of the thesis.

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List of Abbreviations

(RT)-PCR	reverse transcription –polymerase chain reaction
5-FC	5-fluorocytosine
ATCC	American Type Culture Collection
AR	androgen receptors
BLCR UK	Beatson Laboratories Cancer Research UK
BUdR	5-bromo-2'-deoxyuridine
CD	cytosine deaminase
CI	combination index
CMV	Cytomegalovirus
CPM	count per minute
D	Dose
DHT	Dihydrotestosterone
D _m	dose required to produce median effect
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DRE	digital rectal examination
EBRT	external beam radiation therapy
EF	enhancement factor
f _a	fraction affected by the dose
FACS	fluorescence assay cell sorter
FBS	foetal bovine serum
FIAU	5-iodo-2'-fluoro-2'-deoxy-1-β-D-arabino-furonosyluracil
f _u	fraction unaffected by the dose
g	Gram
GAP-DH	glyceraldehyde-3-phosphate dehydrogenase
GCV	Ganciclovir
GM-CSF	granulocyte-macrophage colony-stimulating factor
GnRH	gonadotropin hormone-releasing hormone
h	Hour
HAT	0.1 mM hypoxanthine, 400 nM aminopterin, 0.16 mM thymidine
HGPRT	hypoxanthine-guanine phosphoribosyl transferase

HRPC	hormone-refractory prostate cancer
HSV1-TK	herpes simplex virus type 1 thymidine kinase enzyme
HSV1- <i>tk</i>	herpes simplex virus type 1 thymidine kinase gene
IC ₂₅	drug concentration that inhibits growth of 25% of cells or clones
IC ₅₀	drug concentration that inhibits growth of 50% of cells or clones
IGF-1	insulin-like growth factor -1
IUdR	iododeoxy uridine
KBq	Kilobecquerel
l	Litre
LET	linear energy transfer
LH	leutinising hormone
LOH	loss of heterozygosity
m	Milli
mAbs	monoclonal antibodies
MBq	Megabecquerel
MEM	minimum essential medium
MIBG	meta-iodo-benzyl guanidine
min	Minute
MML-V RT	moloney-murine leukaemia virus reverse transcriptase
MRI	magnetic resonance imaging
MTT	3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide
NAT	noradrenaline transporter
PCR	polymerase chain reaction
PET	positron emission tomography
PIN	prostatic intraepithelial neoplasia
PSA	prostate specific antigen
PSMA	prostate specific membrane antigen
RNA	ribonucleic acid
rpm	round per minute
s.d.	standard deviation
SF	surviving fraction
SPECT	single photon emission computerised tomography
TCA	trichloroacetic acid
TdR	thymidine

TK	thymidine kinase
TRC	targeted-radiation cytotherapy
TRUS	transrectal ultrasound
TURP	transurethral resection of prostate
UV	Ultraviolet
V	Volt
□	Micro

Abstract

The strategy of suicide gene therapy in cancer is based on the idea of enabling tumour cells, by gene transfer, to convert a non-toxic pro-drug into a toxic product. Previous work has shown that the combination of herpes simplex virus type 1 thymidine kinase gene (HSV1-*tk*) transfer with the pro-drug ganciclovir (GCV) to be a promising suicide gene therapy in cancer. Unlike several other gene therapy systems, early-phase clinical trials of this strategy have shown encouraging results. Therefore, methods to improve its therapeutic efficacy are urgently sought.

The thymidine analogue 5-iodo-2'-fluoro-2'-deoxy-1-β-D-arabino-furonosyluracil (FIAU) is an alternative substrate of the HSV1-TK enzyme. The iodine atom of FIAU can be substituted with radioactive iodine, for example; [¹²³I]-iodine, and thereby utilised for the delivery of ionising radiation into tumour cells expressing the viral *tk* gene. The aim of this study was primarily to investigate the therapeutic potential of combining HSV1-*tk* gene transfer and [¹²³I]FIAU for the targeted radiation cytototherapy of prostate cancer cells alone or in combination with GCV.

The HSV1-*tk* gene was cloned into the plasmid vector pcDNA3.1. This plasmid, driven by the ubiquitous promoter of CMV, was then used to transfect the prostate cancer cell line DU145 and the glioma cell line UVW. A viral TK positive, commercially available cell line derived from osteosarcoma (143B-TK) along with its TK-negative clone were also used for comparison.

The viral *tk* gene transfection efficiency was assessed by three independent methods. Firstly, the uptake of [¹²³I]FIAU normalised to the uptake of tritiated thymidine ([methyl-³H]TdR); secondly, GCV sensitivity, assessed by the MTT assay; and thirdly, by the detection of HSV1-*tk* gene by RT-PCR. The highest specific activity of [¹²³I]FIAU was obtained by the use of a no-carrier added method of synthesis.

The cytotoxicity of [¹²³I]FIAU was assessed by clonogenic assay after incubating monolayers of parental and TK-positive clones of the cell lines with a range of doses of

[¹²³I]FIAU for the periods of 4 h, 8 h and a period equal to their doubling times. The effect of this treatment on cell cycle progression was assessed by FACS analysis after staining the cellular DNA with propidium iodide.

Combination therapy using GCV and [¹²³I]FIAU for the treatment of TK-positive clones of the prostate cancer cell line DU-145 and the osteosarcoma cell line 143B was assessed by the method of median effect analysis and combination index. Monolayers were treated with a constant ratio of various doses of [¹²³I]FIAU for 4h or GCV for 72h. The combination therapy followed three different timing schedules of GCV-before-[¹²³I]FIAU, [¹²³I]FIAU-before-GCV, or simultaneous therapy.

The expression of HSV1-*tk* gene by the three cell lines was confirmed by the three methods described above. For instance, the TK positive clone of the cell line DU145 exhibited 4.25 ± 0.15 times higher [¹²³I]FIAU/ [methyl-³H]TdR uptake ratio and 43 times higher sensitivity to GCV compared with the parental cell line.

The three cell lines demonstrated sensitivity to radiolabelled FIAU, which was significantly enhanced by HSV1-*tk* gene expression. This sensitivity was time-, dose-, and proliferation-dependent. Maximum cell kill was achieved when the monolayers were exposed to [¹²³I]FIAU for a period equivalent to the cellular doubling time. For example, the sensitivity enhancement factor by *tk* gene expression of the cell line DU145 increased from 5.2 to 7.6 when the treatment period was prolonged from 4 h to 26 h (doubling time of DU145).

Following the treatment with [¹²³I]FIAU for a period equal to the doubling time, cells were arrested at G2/M phase of the cell cycle. For instance, 49% of DU145-TK cells treated with 1 MBq/ml for 26 h were at G2/M phase compared with 21.9% of the untreated cells. In contrast, incubation of DU145-TK or 143B-TK cell lines with lethal doses of [¹²³I]FIAU for 4 h and GCV for 72 h had no significant effect on cell cycle progression.

Comparison of the effectiveness of [¹²³I]FIAU in the monolayer and spheroid cultures indicated that clonogenic cell kill resulting from Auger electron bombardment was restricted to targeted rather than bystander cells.

The combination therapy of [¹²³I]FIAU and GCV of the cell line DU145-TK resulted in antagonistic effect throughout the examined dose range of the schedules of FIAU-before-GCV and simultaneous therapy and the low toxicity concentration range (lower surviving fractions) of the GCV-before-FIAU schedule. The high toxicity concentration range of the latter schedule has shown evidence of additive effect. For the osteosarcoma cell line 143B-TK, synergistic effect was observed at the high toxicity concentration range of the three combination schedules and antagonism at the low toxicity concentration range of the combinations.

We concluded from this *in vitro* study that the combination of HSV1-*tk* gene transfer and the delivery of radiolabelled FIAU is a promising strategy for targeted radiation cytototherapy of prostate cancer. This proliferation-dependent therapy has caused significant cell cycle arrest that warrants further investigation. Furthermore, the combination of GCV and radiolabelled FIAU for the treatment of tumour cells expressing the gene of viral TK resulted in a dose- and schedule-dependent synergism. We believe that these encouraging results should be substantiated by *in vivo* experiments in the near future.

Chapter 1 : General introduction

1.1 Prostate cancer epidemiology and risk factors

1.1.1 Incidence

There were 679,000 new cases of prostate cancer worldwide in 2002, making this the fifth most common cancer in the world and the second most common in men (11.7% of new cancer cases overall; 19% in developed countries and 5.3 % in developing countries) (Parkin et al., 2005). In comparison with the previous global estimates this shows an increased incidence of prostate cancer (Parkin et al., 1999).

Prostate cancer is a disease of the elderly. Around the world, three quarter of cases occur in men aged ≥ 65 years. It is therefore more common in countries with a higher proportion of elderly men in their population (Parkin et al., 2001). Prostate cancer is unique among solid tumours in that it exists in two forms: a histologic or latent form, which can be identified in approximately 30% of men older than 50 years and 60% to 70% of men older than 80 years, and a clinically evident form which affects approximately one in six American men. Latent prostate cancer is believed to have similar prevalence worldwide and among different ethnicities, whereas the incidence of clinical prostate cancer varies dramatically between and within different countries (Reiter & deKernion, 2002).

Incidence rates are now influenced by the diagnosis of latent cancers by screening asymptomatic individuals, so that where this practice is common, the incidence may be very high (124.8 per 100,000 in United States, for example, where it is now by far the most commonly diagnosed cancer in men (Parkin et al., 2005).

1.1.2 Mortality

The prognosis is relatively good; prostate cancer is a less prominent cause of mortality with 221,000 deaths in 2002 (5.8 % of cancer death in men and 3.3% of all cancer deaths) (Parkin et al., 2005). Mortality is affected by survival and survival is significantly better in high-risk countries, but much of this is a consequence of more latent cancer being detected by screening procedures. As a result, mortality rates are probably a better guide to the risk of invasive prostate cancer in different populations.

1.1.3 Risk Factors

Although the specific causes of prostate cancer initiation and progression are not yet known, considerable evidence suggests that both genetics and environment play a role in the evolution of this disease. The most convincing evidence for the role of the environment comes from migration studies showing an increased incidence of prostate cancer in first generation US immigrants from Japan and China (Muir et al., 1991; Shimizu et al., 1991).

1.1.3.1 Age, race and family history

Age is a strong risk factor for prostate cancer, white US men aged 75-79 years have approximately 130 times the risk of men aged 45-49 years (Parkin et al., 1997). Numerous genetic alterations have been demonstrated in prostate cancer tissue, suggesting that cumulative damage to DNA with age may partially explain this trend (Chan et al., 1998c).

Race is one of the few well-established risk factors for prostate cancer incidence and mortality. In the US, the disease is 66% more common and twice more likely to be fatal among African-Americans than Caucasians (1997). In contrast, blacks in Africa have some

of the lowest rates of prostate cancer worldwide, although this may partially due to incomplete disease recording (Parkin et al., 1997). Japanese and Chinese men appear to have lower rates of prostate cancer than their ethnic counterparts.

A number of case-control studies have demonstrated familial clustering of prostate cancer, suggesting that some prostate cancers may be inherited (e.g. (Carter et al., 1992a; Carter et al., 1993; Carter et al., 1991; Steinberg et al., 1990). One study of 1085 prostate cancer patients found that a history of prostate cancer in any first or second degree relative was associated with a threefold excess risk. Risks tend to rise with the increasing number of relatives with a history of prostate cancer, especially if they had cancer at a young age (Carter et al., 1992a).

1.1.3.2 Hormones

Several lines of evidence support a role for sex hormones as initiators or promoters of prostate cancer (Wilding, 1995). Prostate tumours are exquisitely sensitive to androgens and regress after medical or surgical castration. Men castrated at an early age rarely experience prostate cancer (Hovenanian & Doming, 1948) and prolonged administration of high level of testosterone can produce prostate cancer in rats (Noble, 1977; Pollard et al., 1982).

Young African American men have 15% higher total circulating testosterone level than white men (Ross et al., 1986). Therefore, the high incidence of prostate cancer observed in African Americans may be related to the elevated levels of circulating androgen. A meta-analysis of previously published studies on hormonal predictors of prostate cancer concluded that men whose total testosterone level is in the highest quartile are 2.34 times more likely to develop prostate cancer (Shaneyfelt et al., 2000).

1.1.3.4 Diet

Fat consumption has long been suspected to be a risk factor for prostate cancer and prostate cancer mortality rate correlates highly with the average level of fat consumption around the world (Reiter & deKernion, 2002). Fat-free diet has reduced the growth of androgen-dependent tumours in the Dunning model of prostate cancer (Clinton et al., 1988). Other studies have shown that a high-fat diet promotes the growth of LNCaP prostate cancer cells (Aronson et al., 1999; Wang et al., 1995). Gann et al reviewed several mechanisms whereby fatty acid levels may influence prostate cancer (Gann et al., 1994): first, fat can act as a precursor to eicosanoids, which play a critical role in tumour cell proliferation, immune response invasion and metastasis; second, membrane phospholipids fatty acid composition can affect permeability and receptor activity; third, low levels of polyunsaturated fatty acids can inhibit 5 α -reductase activity; and fourth, free-radical formation from fatty acid oxidation can damage DNA.

A number of studies have found an association between high calcium consumption and increased prostate cancer risk (Chan et al., 1998a; Giovannucci et al., 1998; Rodriguez et al., 2003). Chan et al found that calcium intake was an independent predictor of prostate cancer risk (RR, 1.91). Although the mechanism by which calcium could increase prostate cancer risk is not known, it is hypothesised that high levels of calcium may down-regulate vitamin D production, thereby promoting cell proliferation (Chan et al., 1998a).

Lycopene is a carotenoid present at high concentrations in tomatoes. Lycopene is a potent antioxidant which has been studied extensively as a potential negative risk factor for cancer (Di Mascio et al., 1989). In the health Professional Follow-up Study, high lycopene intake was related to a 21% lower risk of prostate cancer. Tomato sauce, the strongest dietary

predictor of reduced prostate cancer risk in the cohort (36% for high versus low intake) was also the major predictor of serum lycopene levels (Giovannucci et al., 1995).

Selenium is a trace mineral component of the antioxidant glutathione peroxidase. The most provocative evidence linking selenium to prostate cancer risk comes from a prospective randomised study to determine if selenium supplementation could reduce the risk of skin cancer (Clark et al., 1998). Although the trial found no influence of selenium on skin cancer, it did incidentally find a dramatic reduction in the incidence of prostate cancer.

1.2 Aetiology and Molecular genetics of prostate cancer

Although only approximately 10% of prostate cancers are believed to be inherited by rare, highly penetrant genes, it is more likely that multiple common low-penetrance genes also contribute to prostate cancer development (Carter et al., 1992a). These genes may predispose to prostate cancer by modulating the response of the host to certain environmental factors or by interacting with other genes.

1.2.1 Inherited susceptibility to prostate cancer

Inherited forms of prostate cancer tend to develop at an earlier age compared with sporadic cases, although differences in terms of biological potential between inherited and sporadic forms of disease are less apparent (Bova et al., 1999).

1.2.1.1 Hereditary prostate cancer genes

A genome wide scan of prostate cancer families found strong evidence for a prostate cancer susceptibility locus on chromosome 1q24-25 (Smith et al., 1996). This region was designated **Hereditary prostate cancer 1 (HPC1)** and it is the most extensively studied

prostate cancer predisposition locus to date. Recombination mapping has localised the gene encoding **2'-5'-oligoadenylate (2-5A) dependent ribonuclease L (RNASEL)** to the critical region of 1q24-25, thus implicating this gene as a candidate for HPC1. At least two mutations inactivating RNASEL have been identified that are potentially responsible for prostate cancer cases in families showing linkage to HPC1 (Carpten et al., 2002).

A number of germline variants and mutations have also been identified in a gene located in chromosome 17 designated **HPC2/ ELAC2**. Variant alleles of ELAC2 were found to be associated with increased risk of prostate cancer (Tavtigian et al., 2001).

Another candidate susceptibility gene for prostate cancer is the macrophage scavenger receptor 1 gene (**MSR1**) located in chromosome 8p22. Family based linkage and association tests have demonstrated that mutations in MSR1 co-segregate with prostate cancer cases in men of African and European descent (Xu et al., 2002).

Another gene called **BRCA2** has been implicated as a prostate cancer susceptibility locus primarily due to its analysis in breast cancer families which suggests that male carriers of mutations are at an increased risk for prostate cancer, particularly at an early age (1999).

CHEK2 is a gene encoding an upstream regulator of p53 in the DNA damage signalling pathway. Recently, studies of CHEK2 showed germline mutations in patients with sporadic and hereditary prostate cancer (Dong et al., 2003). Importantly the majority of CHEK2 mutations identified were not detected in 423 unaffected men, suggesting a pathological effect of CHEK2 mutations in prostate cancer development.

1.2.2 Genetic Polymorphism

Molecular epidemiologic studies have uncovered a number of intriguing associations between specific gene polymorphisms (i.e., inherited sequence variants of a given gene that may alter its function) and risk of developing prostate cancer (Reiter & deKernion, 2002).

1.2.2.1 Androgen receptors

Androgen receptors (AR) mediate testosterone and dihydrotestosterone (DHT) activities by initiating transcription of androgen-responsive genes. The AR gene contains two polymorphisms that may play a role in prostate cancer initiation and progression. Exon 1 contains a polymorphic trinucleotide (CAG)_n repeat encoding polyglutamine (Kantoff et al., 1998). The length of the polymorphism is inversely related to transcriptional activity of the AR gene (i.e., it modulates the response of AR to androgens) (Wieacker et al., 1998).

Giovannucci and associates (1997) reported that men with CAG repeat length less than 18 had a 1.5-fold relative risk (RR) of prostate cancer compared with those with repeat lengths greater than 26. Shorter CAG length were associated with a higher risk of advanced stages (RR, 2.2) and high-grade (RR, 1.9) disease (Giovannucci et al., 1997).

1.2.2.2 5 α -Reductase type 2 enzyme

5 α -Reductase type 2 enzyme converts testosterone to the more potent DHT. It has been hypothesised that its gene polymorphisms that confer a greater degree of enzymatic activity might be associated with increased risk of prostate cancer (Henderson & Feigelson, 2000; Ross et al., 1998).

1.2.2.3 Vitamin D receptor

1,25-dihydroxyvitamin D (1,25D), also known as calcitriol, is the active form of vitamin D. 1,25D has been shown to promote differentiation and inhibit the proliferation and invasive

potential of human prostate cancer cells. Prostate cells express vitamin D receptors (VDR), which mediate the function of 1,25D. Ingles and associates (Ingles et al., 1997) found that the presence of a single “long” poly A allele (i.e., L) conferred a 4.6-fold increased risk of prostate cancer.

1.2.2.4 Insulin-like growth factor -1

The insulin-like growth factor -1 (IGF-1) is part of the IGF axis, which consists of IGF hormones, IGF binding proteins (IGFBP), IGF receptors, and IGFBP protease. IGFs play important roles in cell proliferation and metabolism (Cohen et al., 1994). IGF-1 is one of the strongest risk factors identified to date for prostate cancer (Chan et al., 1998c). Two case controlled studies in Greece (Mantzoros et al., 1997) and Sweden (Wolk et al., 1998) found an association between circulating IGF-1 levels and prostate cancer risk. Results from these retrospective studies were confirmed in the prospective Physician Health Study, in which a direct association was found between IGF-1 levels measured in samples collected before subsequent development of prostate cancer (Chan et al., 1998b). Men with IGF-1 levels in the highest quartile had a 4.3-fold higher risk of prostate cancer compared with men in the lowest quartile. Together these studies provide provocative evidence that IGF-axis plays an important role in the development of prostate cancer.

1.2.3 Somatic gene alterations in prostate cancer

Loss of heterozygosity (LOH) involving chromosome 8p is perhaps the most common deletion event in prostate cancer (Gonzalzo & Isaacs, 2003). LOH of chromosome 8p is believed to be an early event in prostate carcinogenesis that is found in prostatic intraepithelial neoplasia (PIN) and also in prostatic inflammatory atrophy (PIA) lesions.

The existence of a tumour suppressor locus on 8p is supported by LOH and a comparative genomic hybridisation study that demonstrated loss of 8p in approximately 80% of metastatic prostate cancers (Cher et al., 1996).

LOH at chromosome 10q is frequently associated with metastatic prostate cancer. The **Phosphatase and tensin homologue (PTEN)** gene is located in chromosome 10q23 and is frequently mutated or deleted in prostate cancer cell lines and tumours (Li et al., 1997). Absent PTEN expression has been correlated with higher Gleason score and advanced stage of prostate cancer (McMenamin et al., 1999).

The CDKN1B gene is located on chromosome 12p12 encodes **the cyclin-dependent kinase inhibitor p27**. The p27 protein regulates cell cycle progression from G1 to S phase by its inhibitory interaction with the cyclin E/cdk2 complex. Decreased p27 is common in early stage invasive prostate cancers and it has also been associated with an increased risk of biochemical recurrence in patients following radical prostatectomy (Cote et al., 1998). Expression of p27 in prostate biopsy specimens was found to be predictive of expression levels in radical prostatectomy specimens and it correlated with Gleason score and final pathological stage (Thomas et al., 2000).

The Retinoblastoma Susceptibility (Rb) gene located on chromosome 13q has been implicated in prostate cancer development. Mutations in Rb and loss of expression have been reported in 20% to 50% of prostate cancers (Cunningham et al., 1996). The lack of Rb expression has also been observed in approximately 22% of prostate cancers, although LOH at Rb was not significantly associated with absent Rb expression (Cooney et al., 1996).

The α -Methylacyl-CoA racemase (AMACR) gene was identified on chromosome 5p13 and found to be consistently up-regulated in prostate cancer. Approximately 88% of prostate cancer cases analysed by immunohistochemistry demonstrated higher AMARC staining than normal prostate tissue (Luo et al., 2002). An evaluation of AMACR protein expression in prostate needle biopsy specimens demonstrated 97% sensitivity and 100% specificity for detecting prostate cancer, thus, providing further support for the diagnostic usefulness of this newly discovered marker (Rubin et al., 2002).

Telomerase: Telomeres are repetitive non-coding DNA sequences located at the ends of all eukaryotic chromosomes that shorten with each cell division. The loss of genetic information at telomeres is a fundamental component of the normal aging process.

The enzymatic activity of telomerase functions to maintain telomere length and it has been associated with cellular immortalisation and cancer. Telomerase activity is increased in PIN lesions and prostate cancer but absent or low in benign disease and normal prostate tissue. Prostate epithelial cells within high grade PIN and invasive prostate cancer lesions were found to have significantly shorter telomere lengths compared with adjacent normal epithelial cells (Meeker et al., 2002). Telomere shortening is believed to occur at or before the development of PIN lesions and may precede the up-regulation of telomerase activity that is frequently observed in prostate cancer (Meeker et al., 2002).

Increased **Prostate stem cell antigen (PSCA)** expression has been observed in greater than 80% of prostate cancer specimens, including high grade PIN. (Reiter et al., 1998). Higher PSCA expression is associated with increasing Gleason score, disease stage and progression to androgen independence (Gu et al., 2000b).

The Kruppel-like factors 6 (KLF6) gene located on chromosome 10p encodes a zinc finger transcription factor of unknown function and it has been recently implicated in prostate carcinogenesis (Narla et al., 2001). LOH of KLF6 was observed in approximately 77% of prostate tumours with concomitant mutation of the retained allele occurring in 71% of tumours.

1.2.4 DNA methylation and prostate cancer

DNA methylation is one of the epigenetic events that can effect gene expression without altering the actual sequence of DNA. Abnormal methylation of genes such as those involved with the control of cellular growth or detoxification is believed to have a critical role in the early stage of prostate cancer progression (Lee et al., 1994).

1.2.4.1 The ras association domain family protein 1, isoform A (RASSF1A) gene

Abnormal methylation of the RASSF1A gene is common in other cancers like lung cancer (Dammann et al., 2000). The RASSF1A promoter is unmethylated in normal prostate tissue, whereas RASSF1A methylation has been reported to occur in 60% to 70% of prostate carcinomas (Kuzmin et al., 2002). RASSF1A methylation is also a more frequent event in high grade prostate cancers compared with less aggressive tumours (Liu et al., 2002).

1.2.4.2 GSTP1

Glutathione-s-transferase proteins (GSTPs) represent a superfamily of enzymes responsible for detoxifying a wide range of xenobiotics. These enzymes catalyse the nucleophilic attack of reduced glutathione on electrophilic compounds and they have evolved as a cellular protection system against their toxic effects. Aberrant methylation of regulatory sequences

at the GSTP1 locus is the most common somatic genome alteration reported for prostatic cancer and it represents an early event in neoplastic transformation (Jeronimo et al., 2001; Lee et al., 1994).

GSTP1 methylation occurs in approximately 70% of PIN lesions and in the majority (Greater than 90%) of prostate carcinoma but not in normal prostate tissue and benign prostatic hyperplasia (Lee et al., 1994). Abnormal gene methylation has been associated with poor clinical outcomes in patients with prostate cancer and it may serve as a potentially useful tool for disease diagnosis and prognostication (Maruyama et al., 2002). Recent studies have reported measurement of GSTP1 methylation in cells in the urine and urine sediment after prostatic massage of men with prostate cancer (Cairns et al., 2001; Goessl et al., 2001). Enokida et al. have lately reported a novel method of using methylation score (M score) as a diagnostic and staging biomarker for prostate cancer. For prostate cancer detection the M score had 75.9% sensitivity and 84.1% specificity, which is much higher compared with that reported with PSA. Furthermore, the M score showed significant correlation with clinicopathologic features such as high Gleason score, involvement of seminal vesicle, and higher preoperative PSA (Enokida et al., 2005).

1.3 Pathology of prostate cancer

1.3.1 Prostatic intraepithelial neoplasia

Prostatic intraepithelial neoplasia (PIN) consists of architecturally benign prostatic acini or ducts lined by cytologically atypical cells. The presence of high-grade PIN in prostate biopsy samples prompts repeated biopsy according to several studies. The largest studies reported 23% to 35% risk of cancer on subsequent biopsy (Davidson et al., 1995; Kronz et al., 2001; Schlesinger et al., 2005). More and larger foci of high-grade PIN were found in

prostate glands with cancer compared with those without cancer and the amount of high-grade PIN is proportional to the number of multifocal carcinomas (McNeal & Bostwick, 1986).

1.3.2 Adenocarcinoma

1.3.2.1 Tumor location and volume

In clinical stage T2 carcinoma and in 85% of non-palpable tumours diagnosed on needle biopsy (stage T1c), the major tumour mass is peripheral in location (Byar & Mostofi, 1972; Epstein et al., 1994; McNeal, 1969). In the remaining cases, tumours are predominantly located in the transitional zone. Tumours that appear to be unilateral on rectal examination are bilateral in approximately 70% of cases when examined pathologically. Adenocarcinoma of the prostate is multifocal in more than 85% of cases (Byar & Mostofi, 1972). In general, the size of a prostate cancer correlates with its extent. Extraprostatic extension is uncommon in tumours of less than 0.5 cm². Tumours that are less than 4 cm² uncommonly reveal lymph node metastases or seminal vesicle invasion (McNeal, 1992).

1.3.2.2 Grading of prostate cancer

The Gleason grading system is the most widely accepted grading system for the evaluation of prostatic adenocarcinoma (Gleason & Mellinger, 1974). The Gleason system is based on the glandular pattern of the tumour as identified at relatively low magnification. Cytologic features play no role in the grade of the tumour. Both the primary (predominant) and the secondary (second most prevalent) architectural patterns are identified and assigned a grade from 1 to 5, with 1 being the most differentiated and 5 being the least differentiated. Since both the primary and the secondary patterns are influential in predicting prognosis, their

resultant Gleason sum (Gleason grade or score) is obtained by the addition of the primary and secondary grades.

1.4 Prostate cancer diagnosis and staging

1.4.1 Diagnosis

Prostate cancer rarely causes symptoms until it is advanced. Thus, suspicion of prostate cancer resulting in a recommendation for prostatic biopsy is most often raised by abnormalities found on digital rectal examination (DRE) or by serum prostate-specific antigen (PSA) elevations (Carter & Partin, 2002).

1.4.1.1 Digital rectal examination

Prostate biopsy is recommended for all men who have DRE abnormalities, regardless of PSA level, because 25% of men with cancer have PSA levels less than 4 ng/ml (Carter & Partin, 2002). A recent study by Philip et al, concluded that if patients are appropriately counselled before PSA testing, a DRE may not be essential for patients with a PSA level of 2.5-10 ng/ml (Philip et al., 2005).

1.4.1.2 Prostate specific antigen

Prostate specific antigen (PSA) is a 33 kD serine protease of the kallikreine family that is produced primarily by prostatic luminal epithelial cells (Carter & Partin, 2002). PSA is secreted in high concentrations (mg/ml) into seminal fluid, where it is involved in liquefaction of the seminal coagulum (Lilja, 1997; McGee & Herr, 1988) and it is normally found in low concentration in sera (ng/ml). Most PSA in sera is bound to the antiproteases

alpha 1-antichymotrypsin (ACT) and alpha 2-macroglobulin (MG) (Christensson et al., 1990; Lilja et al., 1991; Stenman et al., 1991).

Since the discovery of PSA (Nadji et al., 1981; Wang et al., 1979) and its detection in the sera of prostatic cancer patients (Papsidero et al., 1980), PSA has become one of the most widely used tumour markers in medicine. Early study performed by Myrtle and Ivor in 1989 established a reference range of 0-4 ng/ml to define normal PSA levels (Myrtle & Ivor, 1989). While several studies have attempted to refine the sensitivity and specificity of these levels, to date, these values remain the standard against which elevations are compared (Catalona et al., 1994b; Catalona et al., 1997; Ellis et al., 2001). Nevertheless, in the absence of prostate cancer, serum PSA levels vary with age, race and prostate volume (Carter & Partin, 2002). Initial examination of the use of PSA as a diagnostic tool of prostate cancer has uncovered its low sensitivity and specificity (Polascik et al., 1999; Reissigl et al., 1995). Methods to improve these values have evolved with the goal of increasing the positive predictive value of PSA in early cancer detection, while limiting the number of unnecessary biopsies.

Firstly, the use of different PSA thresholds depending on age and race (e.g. (Catalona et al., 1994a; Catalona et al., 1997; Oesterling et al., 1993)). The recommended reference range for serum PSA (95th percentile) for men aged 40 to 49 years is 0.0 to 2.5 ng/ml; for 50 to 59 years, 0.0 to 3.5 ng/ml; 60 to 69 years, 0.0 to 4.5 ng/ml and 70 to 79 years, 0.0 to 6.5 ng/ml (Oesterling et al., 1993).

Secondly, the prostate specific antigen density (PSAD), which refers to the value of PSA divided by the volume of the prostate gland. A PSAD of 0.15 or greater has been proposed as a threshold for recommending prostate biopsy in men with PSA levels between 4 and 10 ng/ml and no suspicion of cancer on DRE or TRUS (Bazinet et al., 1994; Seaman et al.,

1993). PSAD of the transitional zone better enhances the specificity of serum PSA for prostate cancer detection in patients with PSA of 4.0 to 10.0 ng/ml (Djavan et al., 1998).

Thirdly, the rate of change of PSA (PSA velocity PSAV) has been found to be specific in predicting the presence of prostate cancer. A velocity of > 0.75 ng/ml per year was 72% sensitive and 95% specific in predicting prostate cancer in men with PSA values under 10 ng/ml (Carter & Pearson, 1994; Carter et al., 1992b).

Fourthly, men with prostate cancer have lower percentage of unbound or free to total PSA in sera (Stenman et al., 1994; Stenman et al., 1991). Cristensson and coworkers found that a free/total PSA cutoff of 0.18 (18% free/total PSA) significantly improved the ability to distinguish between cancer and non-cancer subjects as compared with the use of total PSA alone (Christensson et al., 1993). In two prospective multicenter clinical trials, Complexed or bound PSA was found to be superior to total PSA in discriminating between benign disease and prostate cancer (Djavan et al., 2002; Partin et al., 2003).

1.4.1.3 Transrectal ultrasound-directed prostate biopsy

A number of studies have confirmed the inability of transrectal ultrasound (TRUS) to localise early prostate cancer (Ellis et al., 1994; Flanigan et al., 1994). The major role of TRUS is to ensure accurate wide-area sampling of prostate tissue in men at high risk of harboring cancer (Carter & Partin, 2002). Hodge et al, were the first to report that systematic sampling of the prostate guided by TRUS improved the detection rate of prostate cancer over merely sampling hypoechoic or other lesions (Hodge et al., 1989). This sextant technique was accepted as the standard of care until subsequent literatures has demonstrated it to be undersampling. In one study, for example, when the sextant biopsies were repeated in a single sitting, the initial biopsies revealed cancer in 22% of men while 10% had cancer diagnosed only on the second set of biopsies (Levine et al., 1998). Chen et

al used a computer simulation model of reconstructed radical prostatectomy specimens to compare the ability of biopsy regimens published in the literature to detect prostate cancer. The highest cancer detection rate of 94% was found by using 11-core multisite-directed scheme (Chen et al., 1999).

1.4.2 Staging of prostate cancer

The main goals in staging prostate cancer are to predict prognosis and to rationally select therapy based on the predicted extent of disease (Carter & Partin, 2002). The first clinical staging classification system for prostate cancer was introduced by Whitmore in 1956 and later modified by Jewett in 1975 (Jewett, 1975). The tumour, node, metastases (TNM) system for staging prostate cancer was adopted in 1975 by the American Joint Committee for Cancer Staging and End Result Reporting (AJCC) (Collins, 1975; Wallace et al., 1975) and was modified in 1997 to combine clinical stages T2a and T2b (Flemming et al., 1997). Table 1.1 compares the Whitmore-Jewett and TNM classifications.

Clinical staging refers to the assessment of the extent of the disease determined by DRE, serum tumour markers, tumour grade, and imaging modalities. The determination of local extent of the disease, primarily by DRE, is referred to as the T stage. However, because DRE is subjective and poorly reproducible, both understaging and overstaging are often found when the pathologic extent of disease is correlated with DRE findings (Carter & Partin, 2002).

Pathologic staging after histological examination is more useful than clinical staging in the prediction of prognosis because it can accurately determine tumour volume, surgical margin status, extent of extracapsular spread, and involvement of seminal vesicle and pelvic lymph nodes (Carter & Partin, 2002)..

Table 1.1: Prostate cancer staging systems

TNM			whitmore- Jewett	Description
1997	1992	Description		
TX	TX	Primary tumour cannot be assessed	None	None
T0	T0	No evidence of primary tumour	None	None
T1	T1	Nonpalpable tumour not evident by imaging	A	Same as TNM
T1a	T1a	Tumour found in tissue removed at TUR; 5% or less is cancerous with histological grade ≥ 7	A1	Same as TNM
T1b	T1b	Tumour found in tissue removed at TUR; >5% is cancerous or histological grade >7	A2	Same as TNM
T1c	T1c	Tumour identified by prostate needle biopsy owing to elevation in PSA	None	None
T2	T2	Palpable tumour confined to the prostate	B	Same as TNM
T2a		Tumour involves one lobe or less	B1	Same as TNM
	T2a	Tumours involves less than half of one lobe	B1N	Tumour involves half of lobe surrounded by normal tissue on all sides
		Tumour involves more than one lobe	B2	Same as TNM
	T2b	Tumour involves more than half of a lobe but not both lobes	B1	Same as TNM
None	T2c	Tumour involves more than one lobe	B2	Same as TNM
T3	T3	Palpable tumour beyond prostate	C1	Tumour < 6cm in diameter
T3a	T3a	Unilateral extracapsular extension	C1	Same as TNM
T3b	T3b	Bilateral extracapsular extension	C1	Same as TNM
T3c	T3c	Tumour invades seminal vesicle(s)	C1	Same as TNM
T4	T4	Tumour is fixed or invades adjacent structures (not seminal vesicles)	C2	Same as TNM
T4a	T4a	Tumour invades bladder neck, external sphincter and/or rectum	C2	Same as TNM
T4b	T4b	Tumour invades levator muscle and/or is fixed to pelvic wall	C2	Same as TNM
N(+)	N(+)	Involvement of regional lymph nodes	D1	Same as TNM
None	None	None	D0	Elevated prostatic acid phosphatase
NX	NX	Regional lymph nodes cannot be assessed	None	None
N0	N0	No evidence of lymph node metastasis	None	None
N1	N1	Metastases in single regional lymph node, ≤ 2 cm in dimension	D1	Same as TNM
N2	N2	Metastases in single (>2 but ≤ 5 cm) or multiple lymph nodes with none >5cm	D1	Same as TNM
N3	N3	Metastases in regional lymph node > 5cm in dimension	D2	Same as TNM
M(+)	M(+)	Distant metastatic spread	None	Same as TNM
MX	MX	Distant metastases cannot be assessed	None	None
M0	M0	No evidence of distant metastases	None	None
M1	M1	Distant metastases	D2	Same as TNM
M1a	M1a	Involvement of nonregional lymph nodes	D2	Same as TNM
M1b	M1b	Involvement of bone	D2	Same as TNM
M1c	M1c	Involvement of other distant sites	D2	Same as TNM
None	None	None	D3	Hormonal refractory disease

1.4.2.1 Serum tumour markers

Before PSA was available, prostate acid phosphatase (PAP) was the most often used serum marker for staging prostate cancer. However, a normal enzymatic PAP level is not highly predictive of the absence of extraprostatic disease. Several studies have shown that serum PSA correlates directly with advancing clinical and pathologic stage of prostate cancer (Oesterling et al., 1988; Partin et al., 1990; Stamey et al., 1987). However, in most cases, PSA level alone does not provide accurate enough staging information for individual patients because of overlap of PSA levels between stages (Carter & Partin, 2002). As a general guide, most men (80%) with PSA values less than 4 ng/ml have pathological organ confined disease (Catalona et al., 1997) , two of three men with PSA levels between 4 and 10 ng/ml have organ-confined cancer, and more than 50% of men with PSA levels above 10 ng/ml have disease beyond the prostate. Pelvic lymph node involvement is found in nearly 20% of men with PSA levels greater than 20 ng/ml and in most men (75%) with serum PSA levels greater than 50 ng/ml (Partin et al., 1990; Partin et al., 1993b).

1.4.2.2 Histologic grading

The Gleason system of histological grading has been shown to correlate with the pathologic extent of disease (Gleason & Mellinger, 1974). Numerous studies have shown that the presence of Gleason grade 4 (primary or secondary) or Gleason sum of 7 or greater is predictive of a poorer prognosis (Epstein et al., 1993; Partin et al., 1993a; Stamey et al., 1999).

1.4.2.3 Combined use of clinical data to predict pathologic stage

Staging accuracy of prostate cancer can be significantly enhanced by combining the parameters of local disease extent (T stage), serum PSA level, and Gleason grade from the

prostate biopsy specimen (Humphrey et al., 1991). Probability tables, based on these parameters have been constructed based on large number of men who have undergone radical prostatectomy with precise determination of pathological stage (Partin et al., 1997; Partin et al., 1993b). The information extrapolated from these tables can be useful in counselling men with newly diagnosed prostate cancer.

1.4.2.4 Imaging

Modern imaging modalities such as magnetic resonance imaging (MRI) have been employed to obtain more precise local staging of prostate cancer. This is beneficial particularly for patients with intermediate risk disease and those suspected of having gross extracapsular extension, or seminal vesicle involvement. Recent advances in MRI such as the use of endorectal coil (Nakashima et al., 2004) and spectroscopy (Yuen et al., 2004) have improved its accuracy compared with pathological analysis.

Positron emission tomography (PET) using ^{11}C has been used to evaluate patients for local tumour recurrence and regional metastasis (Fricke et al., 2003).

The ProstaScint (^{111}In capromab pendetide) scan uses a radiolabeled monoclonal antibody targeted to the intracellular epitope of the PSMA molecule. It is approved by the United States Food and Drug Administration (FDA) for the diagnostic imaging and staging of newly diagnosed patients who are deemed high risk for pelvic lymph node metastases (Brassell et al., 2005). One multicentre study reported sensitivity, specificity, and overall accuracy of 75%, 86%, and 81%, respectively (Hinkle et al., 1998).

Whole body skeletal scintigraphy (radionuclide bone scan) is the standard technique for detecting bone metastases from prostate cancer (Gerber & Chodak, 1991; Schaffer & Pendergrass, 1976). However, new evidence suggests that whole body MRI is more

sensitive than bone scan in detecting bone metastases from solid tumours(Lauenstein et al., 2004).

1.4.2.5 Staging lymphadenectomy

Surgical removal and histological examination of the pelvic lymph nodes provide the most accurate staging information relative to pelvic lymph node status. Many investigators advocated that in patients with clinically localised and low risk prostate cancer, lymphadenectomy need not be undertaken (e.g. (Alagiri et al., 1997; Bluestein et al., 1994; Narayan et al., 1994). However, this has been challenged by a recent study based on the gamma probe-guided sentinel lymph node identification in patients with prostate cancer (Wawroschek et al., 1999). In patients with PSA \leq 10 ng/ml and biopsy Gleason score \leq 6, positive lymph nodes were identified by radio-guided surgery in 6.8% (positive biopsies in one lobe) and 10.7% (positive biopsies in both lobes) and therefore, these authors suggest performing an operative lymph node staging, even in low risk disease (Weckermann et al., 2005).

1.5 Treatment of early prostate cancer

In recent years, increasing numbers of patients with prostate cancer have been diagnosed while their disease is still clinically localised (Metlin et al., 1998). Patients with clinically localised disease (T1-3 NX M0) and good life expectancy are generally offered radical prostatectomy or radiotherapy (external beam and/or brachytherapy). However, observation and watchful waiting might be a better option for many patients with prostate cancer, particularly elderly patients with low grade tumours or those with multiple severe co-morbidities. For example, the probability that a patient with Gleason score of 2-4, who has

not been treated, will die of prostate cancer within 15 years is only 4-7% (Albertsen et al., 1998).

1.5.1 Radical prostatectomy

Since it was first described by Hugh Young one century ago (Scott, 1983), radical surgery for prostate cancer has gone through several stages of modernisation, starting from simple perineal approach up till the high tech robotic prostatectomy. Most of the efforts were to minimise the unacceptable morbidity, including urinary incontinence, erectile dysfunction and major blood loss. Undoubtedly, the meticulous work by Walsh and Reiner has made major contribution to the modern anatomical radical prostatectomy, particularly the control of the dorsal venous plexus (Reiner & Walsh, 1979) and the nerve-sparing approach (Walsh & Donker, 1982) to eliminate major bleeding and preserve sexual function, respectively.

A study of 1000 patients treated with radical prostatectomy showed that 10-year biochemical disease-free survival was closely associated with T stage, Gleason score, and PSA concentration. Remarkably, the 10 year cause-specific survival was 95-100% for all patients treated with surgery except for those with Gleason score of 8-10 whose survival dropped to 82% (Hull et al., 2002). Another observation in this study is that whilst 85% of patients with PSA <10 ng/ml had 10-year biochemical disease-free survival, only 46% of patients with PSA >20 ng/ml had similar survival.

Several series demonstrated very low mortality and intraoperative and postoperative events. Less than 10% of patients are left with urinary incontinence. About 31 % and 52% of patients will become impotent after bilateral and unilateral nerve-sparing procedures, respectively (Catalona et al., 1999; Lepor et al., 2001).

Enthusiasm for using laparoscopic surgery in urology has led to the development of laparoscopic radical prostatectomy (Guillonnet et al., 1998; Guillonnet & Vallancien,

1999). Short term studies on disease control and complication rates showed similar results compared to open surgery (Rozet et al., 2005; Stolzenburg et al., 2005). Furthermore, innovative robotic laparoscopic prostatectomy is at the focus of leading prostate surgery centres in the world (Abbou et al., 2000; Abbou et al., 2001).

1.5.2 Radiotherapy of prostate cancer

Radiation therapy, the use of ionising radiation to destroy malignant tissue, has been used in the treatment of the prostate cancer since the 1960s. Radiation acts at the cellular level to cause breakage of the DNA, leading to a loss of reproductive integrity and cell death.

Radiation can be produced by naturally occurring isotopes such as cobalt-60 or artificially created isotopes. In a linear accelerator, high frequency electromagnetic waves accelerate charged particles through a linear tube. The high-energy electron beam can then be made to strike a target to produce high-energy X-rays for treating deep tumours. Radiation can also be introduced into the prostate directly using interstitial implantation of radioactive isotope in the prostate itself, a technique referred to as brachytherapy.

1.5.2.1 External Beam radiation

Conventionally planned radiation therapy of prostate cancer involves using plain film images to define the prostate, its location and the field size and shape. Simulation images are then created using bony landmarks and intravesical contrast. This method has two major disadvantages. First, this approach has been insufficiently accurate to include the total prostate volume thereby leading to tumour underdosing (Ten Haken et al., 1989). Second, the proximity of the prostate to critical normal structures, such as the bladder and rectum

has limited the ability to deliver effective dose levels in excess of 70 Gy by means of conventional radiotherapy techniques (Greskovich et al., 1991; Smit et al., 1990).

1.5.2.1.1 Three dimensional CT-guided treatment planning

Three dimensional conformal radiation therapy (3D-CRT) was developed to correct some of the shortcomings of the conventionally planned radiation therapy. This technique describes any multiple-beam arrangement that uses computer-aided devices to plan and deliver prescribed radiation doses conforming to the three dimensional configuration of the tumour whilst minimising the dose to the normal tissues (Porter & Hart, 1999).

The minimum therapeutic gain with 3D-CRT of reduced morbidity has been confirmed in several studies. In a prospective randomised clinical trial, Dearnaley and co-workers reported a significant reduction in rectal complications after 3D-CRT compared with conventional radiotherapy for patients with early prostate cancer (Dearnaley et al., 1999).

Furthermore, 3D-CRT has allowed the escalation of the dose to the tumour beyond that possible with conventional systems, with a reasonable rate of normal tissue complications (Leibel et al., 1991). In a prospective randomised trial, a conventional dose of 70 Gy was compared with a dose escalation to 78 Gy using 3D-CRT (Pollack et al., 2000). While there was no benefit with dose escalation for patients in the favourable prognosis group (PSA < 10 ng/ml), the trial found very significant benefit for patients in the intermediate and poor groups (PSA > 10 ng/ml or T3 disease).

1.5.2.1.2 Intensity modulated radiotherapy (IMRT)

IMRT is a technological advance in conformal radiotherapy in which there is variation of the quantity of radiation (fluence) across the beam. The potential advantages include more

control of the shape of the high dose envelope, for example to include a concavity around an adjacent critical normal tissue, and also the ability to deliver heterogeneous doses to boost the dose to sub-targets in the tumour volume (Leibel et al., 2002).

Zelevsky and colleagues reported the early toxicity and biochemical outcome in 772 patients treated with a dose of 81 Gy or more using IMRT. At three years, grade 2 or more toxicity was reported in just 4%. The three-year actuarial PSA relapse-free survival rates among patients with low-, medium- and high-risk for biochemical relapse treated with 81Gy were 93%, 84% and 81%, respectively (Zelevsky et al., 2002). Moreover, 3D-CRT has allowed dose escalation to the whole prostate, but IMRT can be used to escalate the dose to other targets also, such as the pelvic lymph nodes and intraprostatic tumour nodules (Nutting et al., 2002).

1.5.2.2 Brachytherapy

Brachytherapy is the targeted delivery of radiation through implants directly to the prostate gland. This is either through permanent implants of iodine-125 (^{125}I) or palladium-103 (^{103}Pd) seeds, or temporary implantation with iridium-192 wires through shallow needles. Modern prostate brachytherapy is performed under TRUS guidance and may be used as monotherapy, as a boost to another primary therapy such as EBRT, or as a primary therapy with another treatment such as EBRT used as a boost, with or without adjuvant/ neoadjuvant androgen deprivation therapy (ADT).

Not all patients with localised prostate cancer are suitable for brachytherapy as a sole modality of treatment. Selection criteria include patients with organ-confined disease who are at low risk of developing metastatic disease (PSA <10 ng/ml, stage <T2a, and Gleason score <7), and those who are likely to have a good functional outcome (Mangar et al.,

2005). Studies have shown that patients with prostate volume of less than 35 cm³ have a relatively low incidence of acute urinary retention and urinary morbidity (Gelblum et al., 1999). Similarly, patients with minimum urinary symptoms, as determined by the International Prostate Symptom (IPS) score, have a low risk of acute retention and prolonged urethritis (Terk et al., 1998).

In a retrospective study, Zelefsky et al, compared the 5-year outcome and morbidity of 3D-CRT versus ¹²⁵I brachytherapy for early prostate cancer. The 5-year PSA relapse-free survival rates for 3D-CRT and brachytherapy groups were 88% and 82%, respectively ($P = 0.09$). Urinary toxicities were more prevalent in the brachytherapy group and subsequently resolved or improved (Zelefsky et al., 1999). Another study by Ragde and colleagues reported a 70% 10-year disease-free survival for 229 patients treated with brachytherapy +/- EBRT, of which 147 received brachytherapy alone (Ragde et al., 2000). These figures are comparable to surgery or EBRT.

1.5.2.3 Combining external beam radiotherapy with androgen deprivation

1.5.2.3.1 Neo-adjuvant hormonal therapy (NHT)

The role of NHT in the management of localised prostate cancer is to reduce the volume of the prostate tumour before definitive therapy. This might result in an additive effect on cell kill and also allows for a reduction in radiotherapy volume by 20-50% (Forman et al., 1995; Zelefsky et al., 1994).

In phase III Radiation Therapy Oncology Group (RTOG) trial 86-10, 471 patients with T2-4 primary tumours treated with combined androgen blockade for 2 months before and continuing during radiotherapy, were compared with a group treated with radiotherapy alone. The most significant difference was seen in a subgroup of 129 cases with Gleason

score 2-6, in which 8-year survival was 70% versus 52% for those receiving radiation alone (Pilepich et al., 2001). It is now widely accepted that patients who receive radical radiotherapy for locally advanced disease should have at least short-term neoadjuvant hormone therapy (Mangar et al., 2005).

1.5.2.3.2 Adjuvant hormonal therapy (AHT)

The rationale for the adjuvant treatment is to eradicate residual tumour clonogens that remain after radiotherapy, either within the prostate or at distant metastatic sites. The survival benefit of the AHT in patients with locally advanced disease has been confirmed in two RTOG trials (Hanks et al., 2003; Pilepich et al., 1997).

In a very recent update of the RTOG trial 85-31, the conclusion was that, in a population of patients with unfavourable prognosis carcinoma of prostate, AHT after definitive radiotherapy was associated not only with a reduction in disease progression but also with statistically significant improvement in absolute survival (Pilepich et al., 2005). The improvement in survival appeared preferentially in patients with a Gleason score of 7-10.

1.5.3 Other novel treatment modalities for early prostate cancer

Several innovative technologies are being evaluated for the treatment of organ confined prostate cancer. These include cryotherapy, high intensity focused ultrasound, interstitial microwave therapy, transperineal radiofrequency interstitial tumour ablation and laser photocoagulation. With the exception of cryotherapy, there is as yet no sufficient evidence to draw conclusions regarding the effectiveness of these new therapies (Hummel et al., 2003).

1.5.3.1 Cryotherapy

Cryosurgical ablation aims to reduce the prostate tumour by the application of sub-zero temperature, administered via the perineum using cryoprobes. Although this treatment modality is not yet widely practised, potential advantages of its use include, focal application with sparing of normal tissues, treatment of cancers that are not amenable to surgical resection, and minimal bleeding (Shinohara & Carroll, 2002). The use of TRUS for real-time monitoring of the freezing process, improved cryoprobes, and better understanding of cryobiology have all contributed to the resurgence of interest in cryosurgery. In addition to treating organ-confined cancer, cryotherapy has been proposed as a treatment for patients with more locally advanced, T3 disease or for patients with residual or recurrent cancer after radiation therapy. The most commonly used cryotherapy systems use either liquid nitrogen or argon as the source of freezing.

Similar to the results of radical prostatectomy or radiation therapy, outcome after cryotherapy for prostate cancer correlates with cancer stage and grade (Shinohara & Carroll, 2002). Size of the prostate gland is another determinant of the outcome and hence a gland larger than 50 cm³ may best be treated with neoadjuvant androgen deprivation to reduce target volume and allow for more effective cryoablation (Shinohara & Carroll, 1999).

1.6 Treatment of advanced metastatic prostate cancer

1.6.1 Endocrine treatment of prostate cancer

Hormonal treatment of prostate cancer was first described by Huggins and Hodges in 1941 where they showed that either castration or use of diethylstilbestrol (DES) is effective in

improving symptoms of prostate cancer (Huggins & Hodges, 1941). In addition to winning the Nobel Prize, this discovery should be credited further for lasting more than 60 years as the first-line treatment for locally advanced and metastatic disease.

The advent of PSA monitoring has led to what is called stage migration and as a result the percentage of patients with metastatic disease at the time of diagnosis of prostate cancer has decreased enormously. However, the role of hormonal therapy in prostate cancer has been reinvented with the emergence of a group of patients with recurrent disease after the failure of definitive local treatment with radical prostatectomy or radiotherapy.

Androgen deprivation or castration is defined by induction of serum testosterone level of <20 ng/ml (Scherr et al., 2003). This can be achieved surgically by bilateral orchidectomy or medically by estrogen agonists, gonadotropin hormone-releasing hormone (GnRH) agonists, GnRH antagonists, and androgen antagonists. The aim of endocrine treatment is palliation rather than cure as it eliminates symptoms in most symptomatic patients. It also prolongs time to clinical progression and possibly prolongs survival, at least in some patients (Shroder, 2002).

1.6.1.1 GnRH agonists

GnRH is a small peptide that is synthesised in the hypothalamus and secreted into the hypophyseal-portal blood circulation in a pulsatile manner. GnRH stimulates anterior pituitary gland via high-affinity receptors to synthesise and release the gonadotropins, leutinising hormone (LH) and follicular stimulating hormone (FSH). The episodic release of endogenous GnRH prevents receptor desensitisation and is mandatory for continuing androgen production. Continuous stimulation of the pituitary with high concentrations of GnRH agonist induces regulatory changes, possibly down-regulation of GnRH receptors (Tieva et al., 2003; Wolff & Lederberg, 1994). This results in receptor desensitisation and

inhibition of LH release, which further inhibits the production of testosterone by the testicles.

The development of Depot GnRH agonists which, unlike endogenous GnRH, are resistant to peptide degradation have revolutionised the treatment of patients with advanced prostate cancer (Tammela, 2004). They are administered by subcutaneous injection at intervals of 1-3 months. There are several GnRH agonists available including goserelin, leuprorelin, buserelin and tritorelin. Co-treatment with anti-androgen should be used during the commencement of GnRH for 2-3 weeks. This is because GnRH initially imposes an increased release of LH production, and therefore, an increase in plasma testosterone levels lasting 1-2 weeks (Bubley, 2001) leading to what is called tumour flare effect.

1.2.1.2 Anti-androgens

Anti-androgens, steroidal and non-steroidal can be used as monotherapy, neoadjuvant therapy prior to radical prostatectomy or radiation therapy, or in combination with castration (Tammela, 2004). Anti-androgens competitively inhibit the binding and interaction of testosterone and DHT to the prostatic androgen receptors.

Non-steroidal antiandrogens also cross blood-brain barrier and block androgen receptors in the diencephalon, the area that is crucial for GnRH production, and the feedback mechanism, which regulates plasma testosterone levels through LH. This leads to an LH increase in endocrinologically intact males and, therefore, testosterone secretion in the testes (Shroder, 2002). Therefore, treatment with nonsteroidal anti-androgens is the only established way to avoid castration in the endocrine treatment of prostate cancer (Tammela, 2004).

Flutamide, bicalutamide and nilutamide are among the recently studied nonsteroidal anti-androgens for the treatment of advanced prostate cancer. Currently, bicalutamide is the best tolerated agent in this group. Bicalutamide offers quality of life benefits in terms of sexual interest, physical capacity and potentially, preservation of bone mineral density (Iversen et al., 2000). Therefore, bicalutamide monotherapy may be a preferred option for younger, sexually active men with locally advanced prostate cancer who wish to optimise their physical activity.

1.6.2 Management of hormonal refractory prostate cancer

Despite the fact that most patients on androgen deprivation demonstrate encouraging initial response, a large proportion of these patients will eventually progress to hormone-refractory prostate cancer. Hormone-refractory prostate cancer (HRPC) is defined as progressively increasing PSA despite castrate range testosterone. The development of hormone-independent state is a categorical and irreversible phenomenon observed in the majority of patients and occurs within an almost predictable time frame after the initiation of androgen deprivation (Eisenberger & Carducci, 2002).

The main cause of hormone resistance is alterations in the androgen receptor (AR) in the form of mutations and/or amplification (Hennequin, 2004). A common site for genetic gain/amplification in prostate cancer is chromosome X, at the AR locus (Koivisto et al., 1997). It has been suggested that increased AR copy number could play a role in androgen-independent tumour progression. In concordance with data obtained with LNCaP cells *in vitro*, genetic profiling of a series of prostate cancer xenografts revealed that the most consistent change during transition to the endocrine-therapy insensitive stage is AR up-regulation, associated with agonism of AR blockers and changes in relative abundance of

co-activators or co-repressors assembled on the promoters of AR target gene (Chen et al., 2004). Another postulated mechanism of hormone resistance is based on the concept that the growth and survival promoting functions of the androgen receptor can be bypassed by alternative signalling pathways, such that the androgen receptor is no longer relevant to disease progression. One such example is up-regulation of the anti-apoptotic gene BCL2 in late-stage clinical samples (McDonnell et al., 1992; Raffo et al., 1995).

1.6.2.1 Second-line hormonal manipulation

At least four independent trials have suggested the benefit of adding second-line anti-androgen, such as flutamide ((Fowler et al., 1995), bicalutamide ((Joyce et al., 1998; Scher et al., 1997) or nilutamide (Desai et al., 2001). Although response rates between 20% and 80% have been reported, the median duration of response is short, ranging between 2 and 4 months (Eisenberger & Carducci, 2002).

For patients who develop HRPc whilst on maximum androgen blockade with the combination of GnRH agonist and anti-androgen, the withdrawal of anti-androgen has been reported to result in a brief decline in PSA level of $\geq 50\%$ in 15 to 30% of patients (Figg et al., 1995; Scher & Kelly, 1993; Small & Srinivas, 1995). The mechanism of this phenomenon has been attributed to alterations in the androgen signalling cascade, including mutations in the androgen receptor, resulting in the anti-androgen behaving as an activator, not an inhibitor of the androgen receptor (Taplin et al., 1995).

Ketoconazole is an antifungal that was found to cause suppression of testicular and adrenal androgen production. Recent studies have suggested the benefit of using ketoconazole after or at the time of androgen withdrawal (Schlesinger et al., 2005; Small et al., 2004).

1.6.2.2 Chemotherapy of prostate cancer

The combination of mitoxantrone with steroids has been approved by the United States Food and Drug Administration (FDA) as an effective regimen for the palliation of painful bone metastases based on 2 randomised phase 3 studies evaluating the difference between mitoxantrone plus steroids versus steroids alone (Kantoff et al., 1999; Tannock et al., 1996). There was no difference seen in survival between the 2 arms in either study. However, there was significant palliative benefit seen with the combination.

Estramustine phosphate, a combination of nitrogen mustard moiety with estradiol, has also been approved by the FDA for use in patients with metastatic prostate cancer (Alexander et al., 1979; Hauser & Merryman, 1984). In addition to its estrogenic activity that can cause castrate levels of testosterone (Ben-Josef et al., 2001), estramustine exerts cytotoxic effects by interfering with the microtubule structure and binding to the nuclear matrix (Hartley-Asp & Kruse, 1986; Tew & Stearns, 1989).

Docetaxel is a semi-synthetic drug belonging to the taxoid family, all of which share the taxane ring. Docetaxel promotes the assembly of microtubules and also stabilises them, preventing their depolymerisation leading to cell death (Ringel & Horwitz, 1991; Rowinsky & Donehower, 1991). Docetaxel as a single agent has been tested in a number of phase 2 clinical trials in metastatic HRPc, with about 40% to 45% of patients having a decrease of $\geq 50\%$ in PSA and objective response rates of about 20% to 40% (Beer et al., 2001; Berry et al., 2001; Friedland et al., 1999).

A phase 2 clinical trial using 5-day regimen of estramustine with docetaxel and low-dose hydrocortisone showed that 30 of 40 patients (68%) had a $\geq 50\%$ decrease in their PSA

levels, and there were 3 complete responses and 9 partial responses in 24 patients with measurable disease (Savarese et al., 2001).

Most recently, two phase 3 trials have demonstrated a clear survival advantage of docetaxel-based chemotherapy for men with HRPC. First, the intergroup study of the Southwest Oncology Group (SWOG 9916) have compared docetaxel and estramustine with mitoxantrone and prednisone (Petrylak et al., 2004). The median overall survival was greater in the docetaxel and estramustine group versus the mitoxantrone and prednisone group (17.5 versus 15.6 months, $P = 0.02$). The median time to progression was 6.3 and 3.2 months, respectively ($P < 0.001$). The PSA response rate was also significantly in favour of the docetaxel and estramustine group (50 versus 27%, $P < 0.001$).

Second, an international, multicenter randomised phase 3 study (TAX327) compared mitoxantrone and prednisone with either docetaxel and prednisone every 3 weeks or weekly docetaxel and prednisone (Tannock et al., 2004). The overall survival was significantly higher in the docetaxel and prednisone every 3 weeks compared with mitoxantrone and prednisone group ($P = 0.009$) but not in the weekly docetaxel and prednisone group ($P = 0.36$). Median survival rates were higher in the docetaxel and prednisone 3 weekly group (18.9 months) compared with the weekly docetaxel and prednisone group (17.4 months) and the mitoxantrone and prednisone group (16.5 months).

1.7 Gene therapy of prostate cancer

1.7.1 Rationale for prostate cancer gene therapy

The available treatments of locally advanced and hormone refractory prostate cancer are so far mostly palliative. Even the lately reported survival advantage of the combination chemotherapy (Petrylak et al., 2004; Tannock et al., 2004) is modest and offers less than two years of disease-specific survival. Therefore, novel treatment modalities are urgently sought. Gene therapy has, undoubtedly, attracted considerable attention as a promising novel approach for prostate cancer.

Gene therapy can be defined as the delivery and expression of genetic information in cells of an individual to restore health or to ease the consequence of a disease (Goncalves, 2005). Cancer gene therapy may be defined as the transfer of recombinant DNA into human cells to achieve an anti-tumour effect. Depending upon the strategy, DNA may be introduced either into cells removed from the body “the *ex vivo* approach” or introduced directly into cells in their normal location “the *in vivo* approach” (Anderson, 1998). Successful gene therapy requires an appropriate therapeutic gene that is delivered efficiently, is expressed therapeutically for a sufficient amount of time with little effect on tissues other than the target tissue, and has few toxic side effects (Foley et al., 2004).

The prostate gland is an ideal organ for cancer gene therapy because it is an accessory organ, easily accessible and it harbours the potential of exploiting several tissue and tumour specific promoters. Moreover, multiple biological processes can be targeted and exploited for gene-based therapeutic approaches (McCormick, 2001), including induction of cytotoxic and apoptotic responses, correction of aberrant growth regulation, and enhancement of anti-tumour immune responses.

1.7.2 Brief history of gene therapy and prostate cancer gene therapy

The potential for treating a patient using gene transfer started not long after Watson and Crick discovered the helical structure of DNA in 1953 (Watson & Crick, 1953). One of the earliest indications that gene transfer might be used therapeutically was proposed from a plant cell experiment in 1968. Roger suggested that: the next step is to build a modified virus...and use the virus to transmit information (Rogers & Pfuderer, 1968).

Before recombinant DNA techniques emerged, the wild type herpes simplex virus (HSV) known to encode a thymidine kinase (TK) activity, was used to convert Ltk⁻ cells from the TK negative to the TK positive phenotype. This was the first instance in which a gene of a known function had been stably acquired by a eukaryotic cell line due to virus infection. Reports on the construction of recombinant DNA appeared in the early 1970s after the finding that linear bacteriophage genomes could be circularised *in vitro* due to the presence of complementary cohesive ends at their termini (Hershey & Burgi, 1965; Watson et al., 1992). Further demonstration of the existence of sequence specific DNA cutters and the isolation of class II restriction endonucleases ((Smith & Wilcox, 1970) and gel electrophoresis technique to dissect large DNA molecules into discrete and manageable fragments (Danna & Nathans, 1971), have led the way to complex libraries of cloned DNA segments containing genes and gene clusters from different species.

Throughout the 1970s and 1980s, similar advances in basic sciences and molecular biology pushed the field of gene therapy to its first clinical trial in 1988 (Culliton, 1988). However, clinical trials to date have been widely unsuccessful and have led many physicians and scientists to doubt the efficacy of this modality (Selkirk, 2004). In 1999, a further set-back to the development of human gene therapy protocols emerged following the first death of a

patient directly related to the gene therapy clinical trial in the University of Pennsylvania (Marshall, 1999; Vogel, 2000).

The reported cure of several young patients with severe combined immunodeficiency was welcomed as the first significant clinical step forward for gene therapy (Cavazzana-Calvo et al., 2000; Hacein-Bey-Abina et al., 2002). However, recent reports have indicated the development of leukaemia syndrome in several of these patients which was discerned to be a direct result of the therapeutic intervention (Hacein-Bey-Abina et al., 2003). This development has again called into question the general safety of the current technology available for gene transfer.

Similarly, gene therapy in oncology has yet to reach phase III trials or show clear cancer patient benefits in large statistically powered phase II trials (Anderson, 1998). Currently, prostate cancer with its unique PSA tumour marker is probably the best model that exists for organ-specific gene therapy. The identification of prostate-specific enhancer/ promoter has allowed gene expression to be confined only to cells that express PSA (Pang et al., 1997; Pang et al., 1995; Schuur et al., 1996).

In addition, The Prostate Expression Data Base (Nelson et al., 2000) has revealed more than 55000 expressed sequence tags (ESTs) from 43 cDNA libraries; of those approximately 500 are prostate unique. The presence of this large number of prostate-unique-promoters and candidate antigens promises to facilitate the design of prostate-specific gene therapy.

The first National Institute of Health (NIH) and FDA approved phase 1 clinical trial of human gene therapy for prostate cancer, was performed by John Hopkins using granulocyte-macrophage colony-stimulating factor (GM-CSF) gene-transduced autologous tumour vaccines (Simons et al., 1999). Running parallel to this trial, a range of other approaches have been investigated preclinically and up to date, to our knowledge, there are

86 approved and/ or ongoing clinical trials of prostate cancer gene therapy worldwide, 8 of them use suicide gene therapy protocols, of which 7 involve HSV1-TK gene transfer. (<http://82.182.180.141/trials/index.html>) (The Journal of Gene Medicine web site)

1.7.3 Strategies for gene therapy in prostate cancer

Current prostate cancer gene therapy research strategies include, in general terms, corrective approaches (replacing deleted or mutated gene) and cytoreductive approaches (immunotherapy and cytolytic/pro-apoptotic) (Mabjeesh et al., 2002)

1.7.3.1 Corrective prostate cancer gene therapy

Corrective strategies may be designed to prevent tumorigenesis in phenotypically normal cells or to induce reversion or regression of transformed cells to non-neoplastic or less transformed phenotype (Mabjeesh et al., 2002). Potential candidate genes for corrective gene therapy could be identified from the wide variety of genetic and epigenetic changes known to occur during the development and progress of prostate cancer (see section 1.2).

p53: The tumour-suppressor gene *p53* encodes a transcription factor involved in regulation of the cell cycle and apoptosis. Asgari et al. have demonstrated the ability of overexpressed *p53* to inhibit the growth of primary cultures derived from radical prostatectomy specimens, surprisingly even when *p53* status is normal (Asgari et al., 1997). This was confirmed on animal models of prostate cancer using a replication defective adenoviral vector encoding CMV driven wild type *p53* gene (INGN 201) (Cowen et al., 2000; Ko et al., 1996). In a recent phase I clinical trial, thirty patients with high risk early prostate cancer were all administered intra-prostatic injections of INGN 201, with no grade 3 or 4

adverse effects, and tumour expression of wild type *p53* induced tumour cell apoptosis (Pisters et al., 2004).

BAX: This is a pro-apoptotic gene, which has been targeted in prostate cancer therapy. Using replication defective adenoviruses encoding BAX, the overexpression of BAX induced therapeutic apoptosis of prostate cancer cells growing *in vivo* and *in vitro* (Li et al., 2001). Honda et al. have shown that BAX induced apoptosis of prostate cancer cells was independent of their Bcl-2 status and androgen sensitivity (Honda et al., 2002).

AR receptor gene: It has been shown that the down regulation of AR receptors using antisense oligonucleotide has caused inhibition of mouse prostate cancer xenografts (Eder et al., 2002). The antisense oligonucleotides inhibit gene expression by binding to a specific target mRNA that has a complementary nucleotide sequence, resulting in degradation and inhibition of translation of the target gene. Another approach using 'decoy' DNA (a double stranded oligonucleotide containing the same DNA sequences as androgen responsive element (ARE) of the AR) was found to have an anti-androgen effect and induce apoptosis in LNCaP cells (Kuratsukuri et al., 1999).

c-Myc: The over-expression of the oncogene c-Myc is frequently observed in advanced prostate tumours (Buttyan et al., 1987; Fleming et al., 1986). Disruption of c-Myc over-expression using antisense oligodeoxynucleotides transduced by a replication-deficient MMTV-promoter-driven retrovirus resulted in a 94% reduction in tumour size of DU145 prostate cancer cell xenografts (Steiner et al., 1998).

BCL-2: This is another oncogene which is frequently overexpressed in androgen refractory prostate cancer (Furuya et al., 1996; McDonnell et al., 1992). A hammerhead ribozyme designed to disrupt bcl-2 expression in LNCaP prostate cancer cells showed pro-apoptotic activity and resulted in increased cell sensitivity to secondary pro-apoptotic agents including phorbol ester (Dorai et al., 1997). BCL-2 antisense oligonucleotide has been

combined with docetaxel in a phase I trial for hormone-refractory prostate cancer (Tolcher et al., 2004). Although dose-limiting toxicity of severe fatigue and severe neutropenia was reported, twelve of the 17 taxane-naïve patients were considered evaluable for response. Seven of these 12 patients had a $\geq 50\%$ reduction in PSA lasting at least 4 weeks.

A potential limitation of corrective gene therapy is the requirement that not even a single neoplastic clonogen must develop. To achieve this goal would require 100% efficiency of gene transfer, as well as sustained transgene expression, or tolerance for repeated vector application (Mabjeesh et al., 2002).

1.7.3.2 Immunotherapy strategies

Tumour cells are poor antigen presenting cells. In prostate cancer in particular, defects in human leukocyte antigen (HLA) system class I expression were observed in 85% of primary and 100% of metastatic tumours (Blades et al., 1995), which implied that the immune system may have an important role in the progression of prostate cancer. Therefore, most of the immunotherapeutic approaches aim to activate the immune system to recognise and act against tumour cell antigens systemically. This might be achieved by enhancing expression of the tumour cell antigen like PSA, or by transfection/ infection of tumour or immune cells with cytokines that stimulate the anti-tumour function of immune cells such as interleukin-2 (IL-2), which stimulates T-cells or GM-CSF which stimulates macrophages and neutrophils.

Cytokines

One means to optimise tumour antigen presentation by gene therapy is through the targeted expression of cytokines in tumour cells. The GM-CSF has emerged as a cytokine with significant efficacy in the induction of an anti-tumour immune response (Dranoff et al.,

1993). GM-CSF may be transduced into autologous or allogeneic tumour cells *ex vivo* using either a retrovirus or other vectors. The transduced tumour cells are then irradiated both to minimise malignant potential and to improve immunogenicity (Simons & Mikhak, 1998). The cells are then re-introduced by vaccination into the patient and tumour response monitored. Phase I clinical trials suggested that both T-cell and B-cell immune response to human prostate cancer can be generated by this treatment (Simons et al., 1999).

Intraprostatic administration of IL-2 gene to patients with localised or locally advanced prostate cancer in phase I clinical trials has shown evidence of systemic immune activation based on an increase in the infiltration of lymphocyte into tumours (Trudel et al., 2003, Beldegrun et al., 2001).

Expression of a tumour antigen

Kim et al. developed a DNA vaccine construct, which encodes for PSA by cloning a cDNA for PSA into a mammalian expression vector under the control of a CMV promoter. PSA specific immune response was induced in mice after immunisation with this PSA vaccine (Kim et al., 1998). There were strong and persistent antibody response against PSA, significant T helper cell proliferation against PSA protein, and T lymphocyte response against tumour cell targets expressing PSA. However, a similar PSA vaccine delivered by modified vaccinia virus has shown low toxic effects and variable efficacy in phase I clinical trials of prostate cancer (Sanda et al., 1999).

In 2004, the Eastern Cooperative Oncology Group reported a phase II clinical trial to evaluate the feasibility and tolerability of a prime/boost vaccine strategy using vaccinia virus and fowlpox virus expressing human PSA in patients with biochemical progression after local therapy for prostate cancer. Out of 64 treated patients, 45% of men remained free of PSA progression at 19.1 months and 78.1% demonstrated clinical progression-free

survival. 46% of patients demonstrated an increase in PSA-reactive-T cells (Kaufman et al., 2004).

1.7.3.3 Cytolytic/ pro-apoptotic strategies

Cytotoxins

Diphtheria toxin A chain (DT-A) was amongst a group of cytotoxins screened using a wide range of prostate cancer cell lines (Rodriguez et al., 1998). This study demonstrated cell cycle- and *p53*-independent cytotoxic activity of DT-A through both apoptotic and non-apoptotic pathways. Regulated expression of DT-A has been achieved both in cultured androgen-independent prostate cancer cells that express the protein BCL-2 and their xenografts using adenovirus vectors (Peng et al., 2002). Furthermore, Zheng and colleagues used human LNCaP prostate xenografts in nude mice to evaluate *in vivo* anticancer efficacy of DT-A gene (Zheng et al., 2003). A single injection of the DT-A gene-encoding lentiviral vector into LNCaP prostate tumours caused complete eradication of approximately 75% of the tumours in the animals.

Oncolytic viruses

Viral vectors (see section 1.7.4.1) may themselves be designed to target and kill tumours without insertion of a foreign transgene. The adenovirus life cycle includes a lytic phase, which can result in host cell death independently of entry into the cell cycle – an important advantage in prostate cancer given the low mitotic rate.

CN706 vector

CN706 is an adenovirus vector with the E1A gene placed under the control of a PSA minimal promoter enhancer. E1A is one of the adenovirus gene products that may function to induce cell death (Rao et al., 1992). In a pre-clinical testing, CN706 infection of PSA-

producing LNCaP cells caused high expression of E1A gene. Furthermore, a single intratumoural injection of the CN706 vector into LNCaP mouse xenograft models destroyed the tumours and abolished PSA production (Rodriguez et al., 1997).

In a phase I clinical trial, twenty patients with locally recurrent prostate cancer following radiation therapy received intraprostatic delivery of a range of doses of CV706 (DeWeese et al., 2001). CV706 was found to be safe and was not associated with irreversible grade 3 or grade 4 toxicity. Five patients from the groups treated with the highest two doses achieved $\geq 50\%$ reduction in PSA.

ONYX-015

The ONYX-015 vector is an adenovirus designed to preferentially replicate in *p53* mutant cells. ONYX-015 was the first genetically engineered replication-competent virus to demonstrate selective intratumoural replication and necrosis in patients (Nemunaitis et al., 2000). In phase II trial, Nemunaitis and colleagues tested intratumoural and peritumoural injection in 37 patients with recurrent head and neck tumours. Significant tumour regression ($>50\%$) occurred in 21% of evaluable patients, whereas no toxicity to injected normal peritumoural tissues was demonstrated (Nemunaitis et al., 2000).

1.7.3.4 Suicide gene therapy

This strategy (also known as gene-dependent enzyme prodrug therapy), involves introduction of a gene encoding a drug metabolising enzyme into target cells, followed by systemic administration of a non-toxic prodrug. The enzyme produced in the target (tumour) cells converts the non-toxic prodrug into a cytotoxic form which then kills the tumour cells (Macrae et al., 2005).

Two of the most commonly used enzyme-prodrug combination in gene therapy protocols are the herpes simplex virus type 1 (HSV1) thymidine kinase (TK) gene used with

ganciclovir (GCV) and the cytosine deaminase (CD) gene used with 5-fluorocytosine (5-FC).

Mammalian cells unlike certain fungi and bacteria do not contain the enzyme CD and therefore do not ordinarily metabolise cytosine to uracil. The introduction of the *Escherichia coli* CD gene into eukaryotic cells allows these cells to produce the toxic antimetabolite 5-fluorouracil from the nontoxic prodrug 5-FC (Mullen et al., 1992).

The efficacy of this system in prostate cancer gene therapy has been studied in a number of preclinical studies. Using subcutaneous LnCaP prostate cancer cells xenografts grown in nude mice, an adenovirus encoding the CD gene was injected directly into the tumour and the prodrug 5-FC was administered intraperitoneally. There was significant reduction in tumour growth compared with untreated tumours (Yoshimura et al., 2002). This strategy of prodrug activation has the advantage of being less dependent on efficient gene transfer than other therapies owing to the bystander effect from the infected/transfected target cells.

In the first clinical trial of its type, the CD gene was combined with *HSV-tk* gene in a replication competent adenovirus (Freytag et al., 2002a). The vector was then administered intra-prostatically to 16 patients with locally recurrent prostate cancer after definitive radiotherapy. Two days later 5-FC and GCV were administered systemically. The drug combination was well tolerated, with few side effects and nearly half of the patients showed a decrease of at least 25% in serum levels of PSA following treatment (Freytag et al., 2002a)

A trimodal gene therapy approach has also been developed, in which replication-competent adenovirus containing a CD/TK fusion gene was administered with FC and valciclovir as well as conventional dose radiotherapy (Freytag et al., 2002a). In the pre-clinical mouse models (DU145 xenografts and LNCaP orthotopic tumours in immune deficient mice), this trimodal approach was found to improve the cure rate and decrease the frequency of

metastases compared with radiotherapy alone. Following this, a phase I clinical trial studied the safety of this strategy for the treatment of newly diagnosed, intermediate- to high-risk prostate cancer patients. There was no dose limiting adverse effects. Patients who received longer treatment with prodrug therapy showed a more prompt decline in serum PSA concentrations (Freytag et al., 2003).

1.7.4 Gene transfer systems

Successful gene therapy requires the development of suitable vectors or vehicles for *in vivo* gene transfer. The alternative *ex vivo* approach of gene therapy is complicated, time consuming, and costly, thus limiting its clinical application. An ideal vector should be safe, stable, easy to produce in large quantities, and capable of achieving efficient and tissue-specific gene expression when directly administered *in vivo* (Li & Ma, 2001). Gene transfer systems are generally classified into viral and nonviral vectors.

1.7.4.1 Viral vectors

The design of viral vectors involves removal of many virally encoded genes (generating so-called gutless vectors) and introduction of the therapeutic gene (Foley et al., 2004). Vectors from retroviruses, adenoviruses, vaccinia virus, adeno-associated virus, and herpes simplex virus are extensively used (Bonnet et al., 2000). Several attributes are considered when choosing a viral vector as illustrated in Table 1.2. The most relevant factors for prostate cancer gene therapy include the ability to transduce non-dividing cells, transduction efficiency and the ability to produce the vector in high titres.

To increase their safety, viral vectors may be designed to be replication deficient, with no further virus particles generated following infection of the target cells. Alternatively the

viral vectors may be replication-competent or replication-attenuated, in which case viral replication can occur in permissive cells (Mabjeesh et al., 2002).

Adenoviruses are one of the most commonly used viral vectors for gene therapy, particularly serotypes 2 and 5 of the subgroup C. Both replication-deficient (e.g. (Herman et al., 1999; Teh et al., 2001)) and attenuated replication-competent (e.g. (Freytag et al., 2002a; Freytag et al., 2003)) forms of adenoviral vectors have been used in the clinical trials of prostate cancer gene therapy. Whilst adenoviruses can transduce both dividing and non-dividing cells efficiently (Bonnet et al., 2000), and high titres can readily be prepared (Herman et al., 1999), there are still major issues of potential immune resistance and inflammatory response.

Hence the only death of a patient undergoing gene therapy was attributed to an immune response to the adenoviral vector (Bostanci, 2002); (Marshall, 1999), adenovirus vectors have been modified extensively to decrease their immunogenicity by deletion of the E1 gene and other genes including E2 and E3 (Herman et al., 1999; Kubo et al., 2003)

Retroviruses integrate into the cellular genome after reverse transcription, allowing long-term and stable gene expression. This integration is beneficial for gene replacement in disorders such as severe combined immunodeficiency (Hacein-Bey-Abina et al., 2002), but it is less important in most strategies of gene therapy for cancer, in which the intention is to kill the target cells. Adverse events have been reported in a clinical trial using retroviral vector, in which two cases of uncontrolled proliferation of T cells were described among patients treated for severe combined immunodeficiency (Hacein-Bey-Abina et al., 2003). Protocols for research on use of retroviruses as vectors account for many trials on gene therapy for cancer, and retroviruses have been used in two clinical trials for prostate cancer (Simons et al., 1999; Steiner et al., 1998).

Vaccinia virus have a wide host range and may transfer large inserts of up to 25 kb, facilitating multi-gene transfer (Peplinski et al., 1998). Vectors derived from vaccinia virus elicit a rapid and sustained humoral immune response. Vaccinia virus vectors have been used in gene therapy for prostate cancer to deliver a vaccine of PSA (Eder et al., 2000; Gulley et al., 2002; Sanda et al., 1999)

Adeno-associated viruses (AAV) are promising vectors for gene delivery because they can integrate into the genome of the host cells (preferentially at chromosome 19 in humans) and persist for the life of the host cell (Muzyczka, 1992). Although no adverse events related to integration of AAV have been reported in clinical trials on gene therapy, it is nevertheless of a potential concern. AAV require additional genes from a helper virus such as adenovirus in order to produce viable viral particles with the packaging cell line (Verma & Somia, 1997). A major drawback for use in some gene therapy protocols is their limited capacity to accept therapeutic genes (Kremer & Perricaudet, 1995).

Table 1.2: Comparison of available viral vectors for gene transfer

Virus (Type)	Infection of non-dividing cells	Transduction efficiency	Capacity for foreign DNA	Concentration ability	Integration into host chromosome	Risk of insertional mutagenesis	Toxicity at high doses	Immune response
<i>Adenovirus (dsDNA)</i>	Yes	High	Small (8 kb)	Can concentrate at high titres	No (lack sustained expression)	No	Yes	Yes
<i>Retroviruses (ssRNA)</i>	No (Except lentiviruses)	Low	Small (<9-10 kb)	Low	Yes (Sustained expression)	Yes	Yes	No
<i>Vaccinia (dsDNA)</i>	Yes	Low	Large (>30 kb)	High	Yes (Transient expression)	NK	NK	Yes
<i>Adeno-associated virus (ssDNA)</i>	Yes (needs helper virus)	High	Small (4.5 kb)	Yes with difficulty	Yes	No	No	Low level
<i>Herpes simplex (dsDNA)</i>	Yes (with or without helper virus)	Moderate	Large (40-150 kb)	High for helper-independent HSV	Latency expression	Yes	Yes	Yes

1.7.4.2 Non-viral vectors

In contrast to viral vectors, non-viral vectors are particularly suitable with respect to simplicity, ease of large-scale production and lack of specific immune response. Recently, several novel non-viral vectors have been developed that approach viruses in terms of transfection efficiency.

Naked DNA

The most technically simple form of gene therapy is the use of naked DNA. A plasmid containing the gene of interest and appropriate promoter is injected directly into a desired site. Administered in this way, the efficiency of transgene uptake/ expression is very low (Mabjeesh et al., 2002). The transforming DNA does not integrate into the host genome and the duration of expression is short. Of all the sites tested so far, the greatest transduction efficiency is observed in muscle (Wolff et al., 1990), where brief low-level expression may be sufficient for eliciting therapeutic immune response.

Attempts to improve the utility of naked DNA plasmid gene transfer include the use of electroporation and gene gun. Electroporation involves exposing cells to a strong electric field that temporarily alters the structure of the cell membrane, facilitating entry of naked DNA. Recent studies have shown that this method facilitates the uptake of plasmid DNA to tissues *in vivo* (Cemazar et al., 2002; Somiari et al., 2000) by applying an electric field to the entire area of interest. Electroporation has been used to deliver transgenes to subcutaneous prostate tumours in mice (Mikata et al., 2002).

Gene guns can be used to propel gold microspheres coated with plasmid DNA into target cells, where DNA is released into the nucleus (Yang et al., 1990). The transfection efficiency is generally low and varies from 1% to 20% depending on the type and depth of the tissue (Johnston & Tang, 1994). In addition to physical trauma leading to loss of

viability of target cells, other disadvantages of this method are short-term low-level expression and shallow penetration of target tissues when the electric pulse is externally applied (Greco et al., 2002).

Liposome vectors and cationic polymers

Liposome vectors are cationic lipids, or more usually a combination of cationic and neutral lipids, which have been used for the cellular delivery of drugs (Ishida et al., 2001). Liposomes are the second most commonly used gene transfer method in current clinical trials. Liposome vectors consist of a DNA plasmid surrounded by a liposomal coat. The positive charge of liposomes facilitates the formation of complexes with DNA and their lipid structure allows them to cross cell membranes.

Liposome vectors are inexpensive and relatively easy to prepare in large quantities. Their large insert capacity permits the transfer of multi-gene cassette of unlimited size. Since they lack proteins, they elicit weaker or no immune/inflammatory responses (Mabjeesh et al., 2002). A disadvantage of liposomal gene transfer is its inefficiency; thousands of liposomes are necessary per cell for successful transduction (Yotsuyanagi & Hazemoto, 1998). Improved formulations, and formation of complexes with targeting molecules have been developed to improve the uptake and decrease the problem of sequestration of liposome vectors in endosomes (Meyer et al., 1998; Otomo et al., 2001; Shi et al., 2001).

Similar to cationic liposome, the cationic polymers bind to nucleic acids to facilitate intracellular delivery. *In vitro*, the dendrimer polyethyleneimine has been used for transfection of prostate cancer cells (Fronsdal et al., 1998).

1.7.4.3 Hybrid Vectors

As a means to circumvent some of the limitations of both synthetic and viral vectors, hybrid vectors combining viral and synthetic approaches have been devised. DNA segments can be complexed to an adenovirus virion with which polylysine or DEAE-dextran has been conjugated (Curiel, 1994; Forsayeth & Garcia, 1994; Wagner et al., 1992). These conjugates improve transfer efficiency by the orders of magnitude in comparison with naked DNA. Similar improvements have been noted for an adenovirus-liposome complex, which resulted in a 1000-fold increase in gene transfer efficiency relative to naked plasmid. (Raja-Walia et al., 1995). The presence of the virion proteins in the DNA-virus complex improves vector attachment and uptake and allows escape of the vector DNA from endosomes prior to fusion with the lysosomes, where they may be degraded (Cristiano et al., 1993).

1.7.5 Prostate-specific gene expression

The efficacy and safety of prostate cancer gene therapy could be improved with the use of prostate-targeted vectors with enhanced transcriptional activity, as well as highly accurate cell-specific discrimination. An effective approach is transcriptional targeting (i.e., restricting expression to prostate-specific tissue by using gene regulatory elements) such as the tissue and tumour specific promoters. This is particularly suitable for use to target tissues of prostate origin because the prostate is one of the organs (in addition to pancreas and breast) that express an unusually high number of unique genes.

1.7.5.1 Tissue specific promoters

Prostate tissue-specific promoters include PSA, the human glandular kallikrein 2 (hK2), prostate specific membrane antigen (PSMA), rodent prostate promoters and osteocalcin.

A minimal composite PSA promoter/enhancer element (PSE) was used to drive expression of Ad E1A in the attenuated replication-competent vector CN706 (Rodriguez et al., 1997; Schuur et al., 1996). The regulation of E1A expression by the PSE element effectively limits cytolytic viral replication to PSA-expressing cells, with resulting therapeutic ratio of between 20:1 and 3000:1 depending upon the cell line tested.

Like PSA or hK3, **hK2** belongs to a large human kallikrein gene family which is located on chromosome 19q13.4 ((Yousef & Diamandis, 2002). DNA transfection and adenoviral transduction studies have confirmed the androgen-responsive and cell specific nature of the hK2 promoter (e.g. (Mitchell et al., 2000). In addition, the increased hK2 expression in carcinoma, as compared to benign tissues, supports its utility as a prostate cancer biomarker (Yousef & Diamandis, 2002).

Although detailed immunohistochemical analysis revealed that PSMA expression is not restricted to prostate tissues alone (Silver et al., 1997), PSMA promoter-driven cytotoxic gene expression was found to be induced by androgen deprivation and is more effective in eradicating androgen-independent prostate cancer cells (O'Keefe et al., 2000).

Osteocalcin is a bone matrix protein produced exclusively by osteoblasts, and its expression is regulated by multiple elements, including the osteo-specific OSE2 element and the vitamin D-responsive element (Hsieh et al., 2002; Lian et al., 1999). Interestingly, primary and metastatic prostate cancer cells were shown to express osteocalcin (Matsubara et al., 2001). Osteocalcin promoter activated adenoviral replication in an oncolytic strategy was

capable of suppressing growth of prostate tumours in animals (Hsieh et al., 2002; Matsubara et al., 2001).

1.7.5.2 Tumour specific promoters

Since no gene has yet been identified that is unique for prostate cancer, gene targeting using prostate tumour-specific promoters is not feasible at the present time. Therefore, it is important to uncover differences in protein and gene expression between normal and cancerous prostate cells to pinpoint genes whose expression is limited to cancer cells.

Telomerase is an enzyme involved in the synthesis and maintenance of chromosome termini that conventional polymerase are unable to replicate fully. Telomerase is composed of an integral RNA component hTERC (Feng et al., 1995) and the catalytic activity is provided by the reverse transcriptase sub-unit hTERT (Nakamura et al., 1997). Both components are necessary for telomerase activity. The expression of hTERT is confined almost exclusively to tumour cells whereas hTERC gene transcription is less stringently limited to malignant cells (Weinrich et al., 1997).

In contrast to most tissue specific promoter elements, the promoter of the telomerase reverse transcriptase gene controls selective expression of telomerase in over 90% of human malignancies, but it is silent in most adult tissues (Kim et al., 1994b). The activity of hTERT promoter was compared with that of the strong ubiquitous cytomegalovirus (CMV) promoter to drive adenovirus vector for the gene of LacZ. In cancer cells, the difference in promoter activity between CMV and hTERT was between 2- and 20-fold, while in normal cells the difference was more than 500-fold. In all cells tested, the hTERT promoter activity was significantly higher in cancer cells than in normal cells, usually by more than 100-fold (Gu et al., 2000a). Wirth et al. (2003) used a telomerase-dependent conditionally replicating adenovirus and demonstrated viral replication in all investigated

telomerase-positive tumour cell lines but not in telomerase negative primary human hepatocytes (Wirth et al., 2003).

1.8 Targeted-radiation cytotherapy (TRC)

1.8.1 Introduction

The dream of magic bullet for cancer therapy describes a therapeutic agent that selectively targets and kills all primary and metastatic malignant cells anywhere in the body without causing significant side effects. Up to date, despite the dramatically improved knowledge of cancer molecular and genetic sciences, this dream is yet to be realised.

Although EBRT retains, beside surgery and chemotherapy, its main role in cancer therapy, significant issues regarding EBRT are still to be addressed. One of the disadvantages of EBRT is that even the most sophisticated methods fail, to a certain extent, to confine the damaging ionising radiation to tumour deposits within the boundaries of the affected organ(s) and spare the normal tissues of the affected organ and the neighbouring structures. This led to the introduction of brachytherapy, whose main objective is to improve the confinement of the ionising radiation within the affected organ. Pre-requisites for this method include easy organ accessibility, certain tumour volume, and most importantly clinically acceptable permanent damage after therapy, which are applicable to localised prostate cancer. However, according to the recent clinical trials comparing brachytherapy with EBRT of prostate cancer, there is no significant survival advantage of brachytherapy compared with EBRT and furthermore, the side effect advantage is still marginal (Ragde et al., 2000; Zelefsky et al., 1999).

Targeted-radiation cytotherapy (TRC) has recently emerged as a promising alternative technique for radiation therapy. TRC selectively limits radiation dose to the targeted cells utilising a specific molecular characteristic or structural or functional alteration of these cells. Potential advantages of this novel strategy include feasibility of systemic administration, selectivity, better dose control and reproducibility. In addition to the established and approved use for thyroid hyperactivity and malignancy, several other applications of TRC are being explored in terms of mechanism of delivery and the type of targeted cells. A number of these applications involve trials to combine TRC with genetic manipulation of tumour cells. However, the majority of these applications are still in their early stages of development. The following is an overview of the available strategies of TRC and their related gene therapy applications.

1.8.2 Monoclonal antibody mediated TRC

Monoclonal antibodies (mAbs) are targeting molecules capable of recognising small differences between molecular groups (epitopes) on the surface of normal and cancer cells (Kohler & Milstein, 1975). Radiolabelled mAbs are becoming more widely used in the field of clinical radio-immunotherapy.

⁹⁰Y-ibritumomab tiuxetan and ¹³¹I-tositumomab are currently the only radiolabelled mAbs approved for treatment of cancer (Sharkey & Goldenberg, 2005). Each is registered for therapy of chemotherapy-refractive, follicular (low grade) non-Hodgkin's lymphoma (NHL), with or without transformation. This strategy uses an antibody that is directed to CD20, an antigen that is abundantly present on a high percentage of both normal and malignant B-cells. In an integrated analysis of 5 clinical trials including 250 patients with refractory or relapsed NHL, response rates ranged from 47% to 68%, complete response

rate ranged from 20% to 38% with a median follow up of 5.3 years (Fisher et al., 2005). Long term side effects reported after radiolabeled mAbs treatment of cancer include hypothyroidism (with ^{131}I products), myelodysplasia, and, possibly, secondary neoplasms (Goldenberg, 2003).

It has been encouraging that significant response to radiolabelled mAbs of NHL patients was observed after the delivery of considerably low absorbed radiation dose (less than 1,000 cGy to a tumour) (Koral et al., 2000; Wiseman et al., 2001). In contrast, a variety of solid tumours in patients with disease burden similar to those treated in NHL trials has failed to elicit responses, even with the delivery of as much as 3,000 cGy to the tumour using ^{131}I - or ^{90}Y -labeled mAbs (Tempero et al., 2000; Tempero et al., 1997).

Prostate cancer is an attractive target for radiolabelled mAbs for numerous reasons including the fact that prostate cancer metastasises almost exclusively to bone marrow and lymph nodes, both of which have good access to circulating mAbs. Furthermore, prostate cancer metastases are frequently small enough for good antibody penetration (Smith-Jones, 2004). Various radiolabelled mAbs are in preclinical and clinical development. They are targeted against a number of different tumour markers, which are either general tumour antigens like TAG72 and CA 170 or prostate specific like PSMA.

The anti-TAG27 mAbs B72.3 and CC49 have been used to target this antigen in prostate cancer patients, initially for radioimmunoguided surgery (Badalament et al., 1993) and as radioimmunotherapy for HRPC (Meredith et al., 1994).

Several radiolabelled mAbs have been developed against PSMA including 7E11-C5 [precursor of ^{111}In -CYT-356 (prostaScint), a FDA approved imaging agent] and J591.

Animal studies of ^{111}In -J591 and ^{90}Y -J591 have demonstrated dose-related increased median survival time relative to untreated controls in nude mice bearing PSMA positive prostate cancer xenografts (Vallabhajosula et al., 2004). In two reported clinical trials using

DOTA-huJ591 conjugates labelled with ^{90}Y and ^{177}Lu , there were dose related antitumour activity including both PSA decline and measurable disease response (Bander et al., 2003).

1.8.3 Receptor-mediated (somatostatin) TRC

Low doses of the radiolabelled somatostatin analogues like ^{111}In -octreotide have been shown to be very useful in detection and staging of the neuroendocrine tumours (Krenning et al., 1989). These compounds have been conjugated with DTPA (diethylene-triamine-pentacetic acid) and more recently with DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) (Otte et al., 1997) as a way of coupling somatostatin analogues with various radionuclides. This strategy of somatostatin receptors (SSRs) targeting with diagnostic radiopharmaceuticals utilises the over-expression of SSRs on the cell surfaces of a number of neuroendocrine tumours (Kwekkeboom et al., 1993). High therapeutic doses of these radiopharmaceuticals can also be given to patients with SSR-positive tumours, a treatment known as peptide receptor radionuclide therapy (PRRT). *In vivo* studies have shown strong growth inhibition of SSR-positive pancreatic liver metastases in rats treated with 370 MBq [^{111}In -DTPA] octreotide. This effect was abolished when the SSR was blocked, indicating that radiolabelled octreotide was internalised specifically by SSR (Slooter et al., 1999). Initial clinical studies with high doses [^{111}In -DTPA] octreotide in patients with metastasised neuroendocrine tumours have been encouraging (Van Eijck, 2005). Most recently, treatment with the newest radio labelled somatostatin analogue, [^{177}Lu -DOTA(0),Tyr(3)] octreotate, which has a higher affinity for the subtype 2 somatostatin receptor, resulted in complete or partial remissions in 30% of 76 patients with inoperable or metastasised neuroendocrine tumours (Kwekkeboom et al., 2005).

1.8.4 Transporter-mediated TRC

The affinity of thyroid tissue for radioactive iodine, a property retained by many thyroid carcinomas, allowing such tumours to be successfully treated by administration of radiolabelled sodium iodide ($^{131}\text{I-NaI}$) was arguably the very beginning of targeted radionuclide therapy. The mechanism mediating iodide uptake across the basolateral membrane of thyroid follicular cells has been clarified by the cloning and characterisation of the sodium iodide symporter, NIS (Dai et al., 1996).

Mandell et al. demonstrated *in vitro* and *in vivo* iodide accumulation in several cancer cell lines, including melanoma, liver, colon carcinoma, and ovarian carcinoma cell lines after transfection with the rat NIS gene (Mandell et al., 1999).

The PSA promoter-directed NIS gene delivery induced tissue specific, androgen-dependent iodide uptake activity of the prostate cancer cell line LNCaP, indicating functional expression of NIS (Spitzweg et al., 2000; Spitzweg et al., 1999). More recently, probasin promoter driven adenovirus encoding human NIS was used to achieve high magnitude and tissue-specific expression of human NIS in prostate cancer cells (Kakinuma et al., 2003). In a preparation for a phase I clinical trial, Dwyer and colleagues injected male dogs intraprostatically with adenovirus-mediated NIS gene followed by i.v. injection of 3 mCi ^{123}I and serial imaging acquisition and then therapeutic dose of ^{131}I . SPECT/ CT fusion images and dosimetry calculations revealed clear images of the NIS transduced prostates and acceptably low doses to other organs. There was no vector-related toxicity or surgical complication observed (Dwyer et al., 2005).

The radiopharmaceutical meta-iodo-benzyl guanidine (MIBG) is a catecholamine closely related to the adrenergic neuron blocking drugs guanethidine and bretylium which is taken

up actively by cells of the sympathetic nervous system and tumours of neuroendocrine origin (neuroblastomas and pheochromocytomas).

Radiolabelled MIBG allows the scintigraphic imaging of neural crest tumours and [¹³¹I]MIBG has been used in the treatment of neuroblastoma and pheochromocytoma (Meller, 1997). The mechanism of uptake and retention of MIBG in tissues and tumours of neural crest origin have been studied extensively.

The cellular uptake of MIBG into adrenomedullary cells and neuroblastoma takes place both by a specific uptake system, known as uptake-one, and by a non-specific mechanism, presumed to be passive diffusion. Uptake-one is an active process by the noradrenaline transmembrane protein (noradrenaline transporter NAT) (Meller, 1997).

The NAT gene has been sequenced (Pacholczyk et al., 1991) and cloned and it has been demonstrated that NAT expression is predictive for MIBG uptake capacity (Mairs et al., 1994). Furthermore, quantification of NAT mRNA could be used for the selection of patients for MIBG therapy (Mairs, 1999). Studies carried out in our department have also demonstrated that the introduction of exogenous NAT gene into non-NAT-expressing tumour cells is a promising method of gene therapy-assisted MIBG-targeted radiotherapy. Amongst the tested cell lines are those derived from glioma (Boyd et al., 2001), prostate cancer (Fullerton et al., 2004) and bladder cancer (Fullerton et al., 2005).

The transfection of prostate cancer cell lines DU145 and LNCaP with CMV or telomerase promoter-driven NAT gene resulted in expression of a functional NAT. This was confirmed by significantly enhanced [¹³¹I]MIBG active uptake and resultant tumour cell kill (Fullerton et al., 2004). Fullerton et al. have also reported 36-fold enhanced uptake of [¹³¹I]MIBG by the bladder cancer cell line EJ138 transfected with telomerase promoter-driven NAT gene,

compared with the parental cell line. The enhanced uptake correlated with dose-dependent cell kill in NAT transfected EJ138 treated with [^{131}I]MIBG (Fullerton et al., 2005).

1.8.5 Bone-seeking TRC

Because advanced prostate cancer often metastasises to bone, bone pain is a common symptom and often determines quality of life in the later stages of disease. Moderate, localised skeletal symptoms often respond to conventional analgesics and/ or single-fraction EBRT. This approach becomes less useful in the context of progressive skeletal metastases, which results in poorly localised or migratory multisite pain.

Selective tumour targeting with bone-seeking radiopharmaceuticals is a suitable option for the management of disseminated disease because it acts systemically and has reduced toxicity compared with other options like wide-field, hemibody radiotherapy. Bone targeting relies on selective uptake and prolonged retention at sites of increased osteoblastic activity. Some therapeutic radionuclides, such as strontium (^{89}Sr) and radium (^{223}Ra), have natural affinity for metabolically active bone. Others including samarium and rhenium, form stable complexes with bone-seeking cations, such as phosphate and diphosphate (Lewington, 2005).

Several studies have demonstrated the efficacy of ^{89}Sr (e.g. (Firusian et al., 1976; Laing et al., 1991; Silberstein & Williams, 1985)). In one retrospective review, 89% of 28 patients reported moderate or good relief of pain (Robinson et al., 1993). Toxicity of ^{89}Sr treatment is limited to temporary myelosuppression, which typically occurs less than 6 weeks after therapy (Lewington, 2005).

1.8.6 Cell cycle targeting (IUdR)

Due to their high proliferation rate compared with normal cells, the DNA of tumour cells can be selectively targeted by incorporating therapeutic nucleosides. Several investigators tested this cycle-specific treatment strategy using the thymidine analogue, iododeoxyuridine (IUdR), labelled with an Auger-emitter radioiodide.

In vitro studies have demonstrated the exquisite toxicity of ^{123}I - and ^{125}I -iodinated IUdR to dividing cells (Kassis et al., 1987; Makrigiorgos GM et al., 1989; Schneiderman & Schneiderman, 1996). Furthermore, the effectiveness of locoregional administration of this agent has been shown in rodent models of gliosarcoma, ovarian cancer ascites, and bladder cancer (Kassis & Adelstein, 1996b). The initial attempt to translate this approach to clinical application was disappointing because of the extremely rapid metabolic degradation of systemically administered radiolabelled IUdR (biologic half-time less than 5 minutes in human (Klecker et al., 1985)).

However, based on the promising results obtained with locoregional administration in animal models, several studies have been conducted to explore this method in patients with cancers suitable for locoregional administration (reviewed in (Bodei et al., 2003)).

The biodistribution of ^{123}I -IUdR has been investigated in one patient with a cystic brain glioma (Kassis et al., 1996). The overall pharmacokinetic pattern of ^{123}I -IUdR administered locoregionally to a human glioma resembles that observed in a rat glioma model, where administration of ^{125}I -IUdR is therapeutically effective (Kassis & Adelstein, 1996a).

A major limitation of this strategy is that, in order to achieve its therapeutic effect, the Auger electron emitter should be incorporated into cellular DNA. This is further complicated by the phenomenon of heterogeneous proliferation in tumours.

Means to circumvent this problem include the use of sustained release biodegradable polymeric implants for controlled release and local delivery of IUdR (Williams et al., 1997) and continuous infusion by micro-osmotic pump. Mairs and colleagues have demonstrated the superiority of micro-osmotic pump over single intralesional injection and slow release biodegradable implants for delivering [^{125}I]-IUdR to rat models of glioma (Mairs et al., 2000). Furthermore, previous studies by our group, using a multicellular tumour spheroid model, have demonstrated that prolongation of the incubation time increased cellular accumulation of [^{125}I]-IUdR (Neshasteh-Riz et al., 1997).

Moreover, it has been hypothesised that benefit may be obtained from the use of both short-range and long-range emitters or by a combination of short-range emitters and EBRT (O'Donoghue & Wheldon, 1996).

1.8.7 Enzyme mediated TRC (FIAU)

Incorporation of Auger electron emitters into DNA is an efficient application of irradiation, capable of inducing cell death with virtually no damage to the surrounding cells. Radiolabelled thymidine (TdR) analogs, such as IUdR, represent the most extensively explored radiobiologic model for cancer therapy with Auger-electron emitters (Bodei et al., 2003). However, as discussed previously, systemic administration of radio-iodinated IUdR is not feasible in the presence of very efficient hepatic dehalogenation (Calabresi et al., 1961; Klecker et al., 1985) leading to a biologic half-life of only a few minutes. Furthermore, cellular retention of this radionuclide relies entirely on its incorporation into DNA, which takes place only during the S phase (DNA synthesis) of cell growth.

In an attempt to overcome these obstacles and utilise this strategy more efficiently, this thesis proposes a novel candidate for TRC with longer biological half-life and additional mechanism of cellular retention.

5-iodo-2'-fluoro-2'-deoxy-1- β -D-arabino-furonosyluracil (FIAU) is a thymidine analogue that has been used as an antiviral agent particularly for chronic hepatitis B virus infection (McKenzie et al., 1995; Tennant BC, 1998). FIAU is more stable than IUdR because of the high chemical and metabolic stability of the N-glycosyl linkage in the pyrimidine nucleosides that contains the 2'-fluoro substituent in the arabinosyl configuration. (Philips et al., 1983)

FIAU has also been radiolabelled and used as a viral infection imaging agent (Saito et al., 1982; Saito et al., 1984; Tovell et al., 1987). FIAU can also be labelled with several different radionuclides, including carbon-11, iodine-131, iodine-123 and iodine-124 (Misra et al., 1986), enabling clinical imaging with positron emission tomography (PET) and single photon emission tomography (SPECT) (Gambhir et al., 2000). Due to its viral thymidine kinase sensitivity and specificity, radiolabelled FIAU can be used as a reporter/ marker probe for the non-invasive imaging of HSV1-*tk* gene expression (Jacobs et al., 1999; Tjuvajev et al., 1996; Tjuvajev et al., 1995).

HSV1-TK, like mammalian TK, phosphorylates thymidine. Unlike mammalian TK, HSV1-TK has relaxed substrate specificity and phosphorylates acycloguanosines, e.g. acyclovir (ACV) (Elion et al., 1977; Fyfe et al., 1978), ganciclovir (GCV) (St Clair et al., 1987) and penciclovir (PCV) (Larsson et al., 1986) as well as TdR analogues e.g. FIAU (Cheng et al., 1981). Cellular enzymes then convert acycloguanosines monophosphates and the monophosphate of FIAU to di- and triphosphates (Furman et al., 1980; Miller & Miller, 1980), which if present in sufficient concentration will kill cells by incorporating as chain

terminating derivatives and/ or inhibition of DNA polymerase (Furman et al., 1979; Furman et al., 1984). In contrast, in mammalian cells without HSV1-TK, TdR analogues are not phosphorylated fast enough to produce toxic concentration of their metabolites. This is because HSV1-TK is more than 1000 times as active at substrate phosphorylation as the endogenous mammalian cellular kinase (Nasu et al., 2000).

In addition to the toxicity resulting from DNA incorporation, FIAU conjugated to radioactive iodine will directly deliver ionising radiation into the nucleus. This will ensure precisely localised radiation dose to tumour cells (Fig. 1.1).

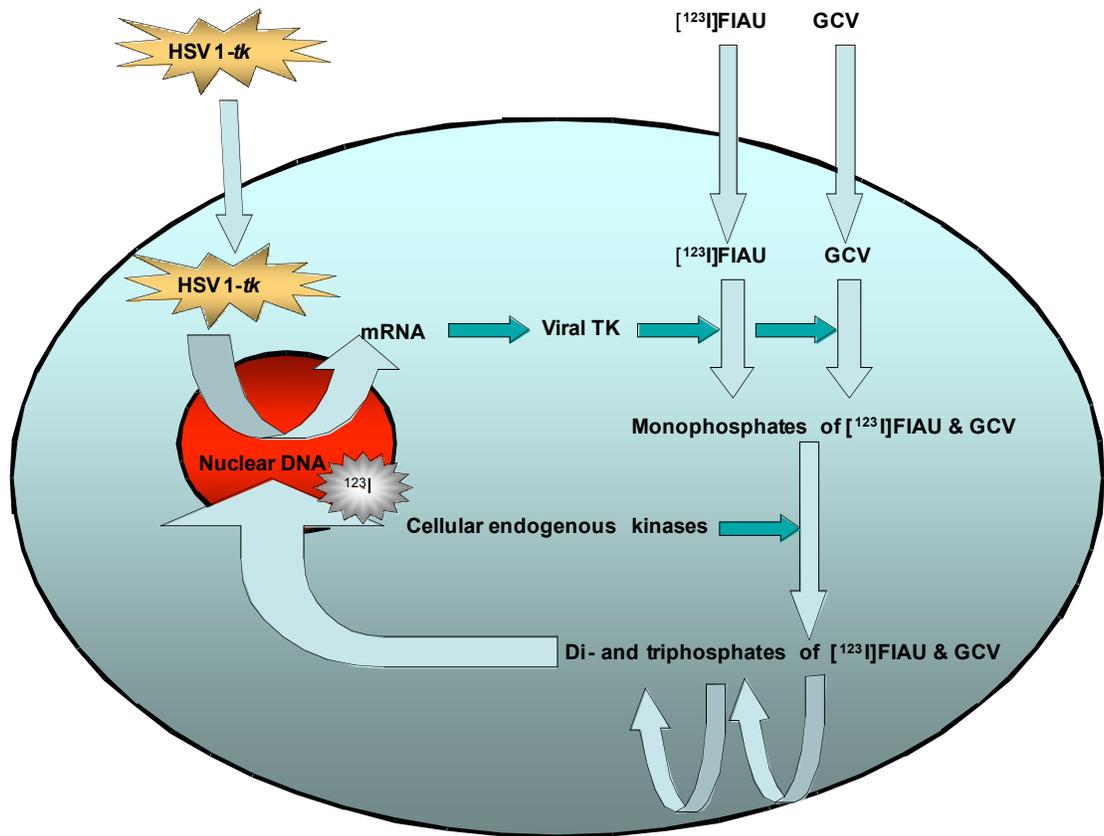
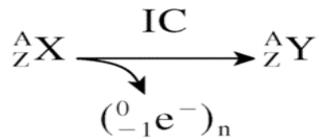
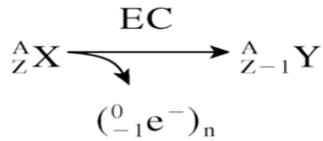


Figure 1.1: Consequences of the introduction and expression of viral thymidine kinase (TK) gene into a mammalian cell followed by exposure to GCV and/ or $[^{123}\text{I}]\text{FIAU}$.

1.8.8 The choice of radionuclide

The Auger electron emitters decay by electron capture (EC) or internal conversion (IC) resulting in a vacancy, most commonly in the K shell.



Such vacancies are rapidly filled by electrons dropping in from higher shells. This process leads to a cascade of electron transition and results in the emission of characteristic X-ray photon or an Auger electron (${}^0_{-1}\text{e}^-$). An atom undergoing EC or IC emits, on average, 5-30 Auger electrons with energy ranging from an eV to approximately 1 keV. The very low energies of Auger electrons have two major consequences. Firstly, these light, negatively charged (-1) particles travel in contorted paths, and their range in water is from a fraction of a nanometer up to approximately 0.5 μm . Secondly, high linear energy transfer (LET) of multiple ionisations (4-26 keV/ μm) occurs in the immediate vicinity of the decay site (Table 1.3).

In contrast, the LET of β^- -particles, which are energetic and negatively charged, is very low (approximately 0.2 keV/ μm) along their up to a centimeter path (i.e., they are sparsely ionising). The energy spectrum of β^- -particles has continuous spectrum ranging from 0 to maximum available energy Q. Subsequently, the use of β^- -particle emitters, like ${}^{131}\text{I}$, as therapeutic agents necessitate the presence of high radionuclide concentration within the targeted tissue (Kassis & Adelstein, 2005). The β^{++} -particles, on the other hand, are

positively charged with mass and charge equal to a helium nucleus, energies ranging from 5-9 MeV, and corresponding tissue ranges of 5-10 cell diameters (Table 1.3).

Table 1.3: General characteristics of therapeutic radionuclides.

(Modified from (Kassis & Adelstein, 2005))

Decay	Particles	$E_{(min)}-E_{(max)}$	Range	LET	Examples
β^- -particle	energetic electrons	50-2,3000 keV*	0.05-12 mm	0.2 keV/ μ m	^{131}I ^{89}Sr
α -particle	He nuclei	5-9 MeV [§]	40-100 μ m	80 keV/ μ m	^{211}At ^{212}Bi
EC/IC (Auger)	Nonenergetic electrons	eV-keV [§]	2-500 nm	4-26 keV/ μ m	^{123}I ^{125}I

* Average (>1% intensity); continuous distribution of energy.

[§]Monoenergetic.

EC = electron capture; IC = internal conversion

The principle target for the biological effects of ionising radiation is DNA resulting in several different lesions. These DNA lesions include steadily repairable lesions like single-strand breaks (SSB), and more difficult to repair lesions of double-strand breaks (DSB) and multiply damaged sites (MDS). These lesions may be produced by direct ionisation of DNA (direct effect) or by the interaction of free radicals (mostly hydroxyl radicals produced in water molecules that diffuse a few nanometers) with DNA, an interaction that may be modified by radical scavengers (Kassis & Adelstein, 2005). The distribution of ionisations within DNA and the type of lesion created depend on the nature of the incident particle and its energy. Low energy electrons like Auger electrons generate clusters of high ionisation density along an irregular path (Fig. 1.2, centre), whereas for energetic β^- -particles ionisations along their linear track are infrequent (Fig.1.2, top). For α -particles,

high ionisation densities occur along a linear track (Fig. 1.2, bottom). Therefore, the high specific ionisations of α -particles and Auger-electron cascade can induce less repairable lesions (e.g. DSB) than those created by the sparsely ionising radiation of β^- -particles.

Furthermore, the cytotoxic efficiency of high LET radiation is not affected by cellular proliferation rate and or the oxygen tension in tissues (Zalutsky & Vaidyanathan, 2000). This is advantageous particularly in slow growing tumours and in the presence of hypoxic regions within the tumour.

The physical properties of the available Auger electron emitters are illustrated in Table 1.4. The modest decrease in DNA breakage efficiency of ^{123}I compared to ^{125}I (smaller number of electrons emitted per decay) might be more than compensated by the advantage of the much shorter half-life. The 60-day half-life of ^{125}I imposes severe limitations in terms of radiation protection (Lobachevsky & Martin, 2004). Therefore, we selected the radionuclide ^{123}I as a conjugate to FIAU for this *in vitro* study.

Table 1.4: Physical properties of Auger-electron emitters (From (Kassis & Adelstein, 2005))

Radionuclide	Average number of electrons emitted per decay	Half-life
^{125}I	20	60.5 d
^{123}I	11	13.3 h
^{77}Br	7	57 h
^{111}In	15	3 d
$^{193\text{m}}\text{Pt}$	30	4.3 d
$^{195\text{m}}\text{Pt}$	36	4 d

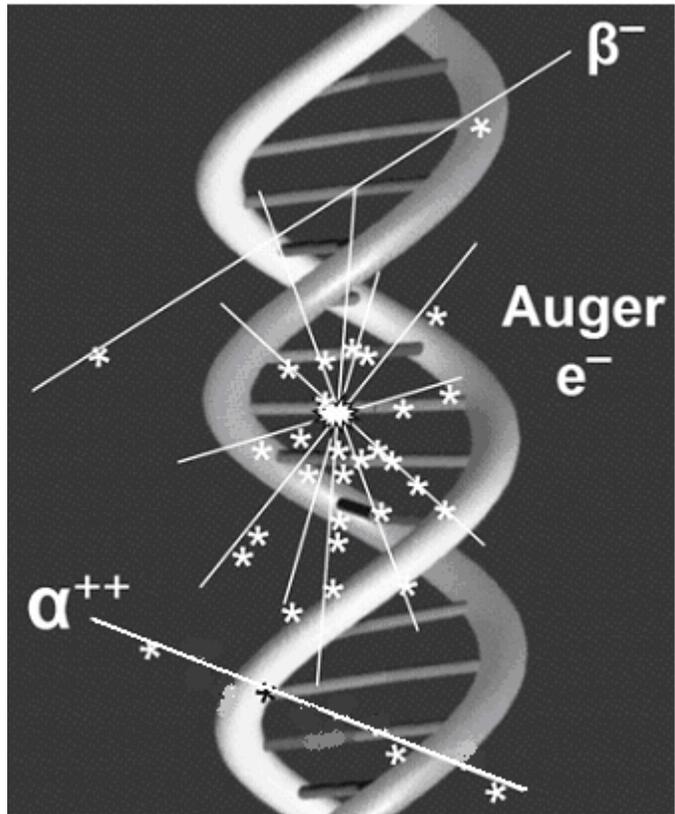


Figure 1.2: Local density of ionisation (*) produced along track (___) of energetic β^- -particles, Auger electrons, and α -particles. (modified from Kassis & Adelstein, 2005)

AIMS OF THE PROJECT

The main objectives of this study were as follows:

- a) To transfect cancer cell lines including those derived from prostate cancer with the HSV1-*tk* gene and then to evaluate HSV1-*tk* gene expression using three different methods.
- b) To test the hypothesis that radiolabelled FIAU can be used for targeted radiation cytotherapy of tumours expressing HSV1-*tk* gene.
- c) To explore the response of tumour spheroids to this new strategy in order to assess the possibility of enhanced response by bystander or cross-fire effects.
- d) To investigate the effect of cellular proliferation rate on the response of TK-positive tumour cells to radiolabelled FIAU.
- e) To determine the cell cycle changes resulting from the treatment of TK-positive tumour cell lines with radiolabelled FIAU and GCV using FACS scan.
- f) To evaluate the combination therapy effect of GCV and radiolabelled FIAU on tumour cells expressing the viral TK enzyme using the method of median effect analysis and combination index.

The ultimate objective of this study was to optimise our basic knowledge of this novel treatment modality in order to develop an approvable clinical trial protocol.

Chapter 2 : General Materials and Methods

2.1 Materials

2.1.1 Cell lines

2.1.1.1 DU145

This cell line was established from tumour cells removed from a metastatic central nervous system lesion of a 69-year-old man with prostate cancer in 1975(Mickey et al., 1977).

The cells are epithelial-like, adherent and grow as monolayers.

2.1.1.2 PC3

This is a human prostate carcinoma cell line which was established from the bone marrow metastasis isolated post-mortem from a 62-year-old Caucasian man with grade IV prostate cancer (poorly differentiated adenocarcinoma) after androgen suppression therapy(Kaighn et al., 1979). These cells are epithelial-like and grow adherently in monolayers or in multilayer foci. This cell line was purchased from the American type Culture Collection (ATCC), Manassas, USA.

2.1.1.3 UVW

This is a human glioma cell line, which was established from an anaplastic astrocytoma of normal adult brain in the Department of Medical Oncology, University of Glasgow(MacDonald et al., 1985). These cells are fusiform and grow in monolayers or multicellular spheroids.

DU145 and UVW were obtained from our departmental maintained cell lines.

2.1.1.4 143B-TK- (abbreviated to 143B)

This is a human osteosarcoma cell line derived from 13-year-old Caucasian female.

These cells are thymidine kinase deficient (TK-) and are resistant to BUdR (Rhim et al., 1975). Their morphology is fibroblast-like.

2.1.1.5 143B PML BK TK (abbreviated to 143B-TK)

This cell line was derived from the human osteosarcoma cell line 143B by the insertion of the PML BK TK plasmid carrying a viral thymidine kinase gene (Manservigi et al., 1988).

The plasmid contained in these cells contains a sequence of the plasmid PML, a derivative of pBR322/BK virus/ herpes thymidine kinase. Deletions created in the viral DNA negate the chance of viral infection.

143B-TK and 143B were purchased from the American type Culture Collection (ATCC), Manassas, USA.

2.1.2 Tissue culture media and supplements

Material	Supplier
Cell culture plastic dishes (all sizes)	Becton-Dickinson Labware, Cowley, Oxfordshire, UK
Sterile plastic pipettes	Corning Incorporated, Corning, New York
Minimum Essential Medium (MEM)	GIBCO, Paisley, UK
PRMI 1640+L-Glutamax	
L-glutamine	
BUdR	
FBS	Sigma, Gillingham, Dorset, UK
HAT	
	GIBCO, Paisley, UK

DMSO	Fisher Scientific UK Ltd, Loughborough, UK
Fungizone (amphotericin)	GIBCO, Paisley, UK
Penicillin/ Streptomycin	
EDTA-Trypsin	
G418 (geneticin)	
Liquid nitrogen	Cryoservice Ltd, Worcester, UK

2.1.3 TK plasmids

pcDNA3.1 TK

This TK encoding plasmid was constructed in our department and transformed utilising E-coli strain DH5 α , see section 2.2.2.2.

PML BK TK

This is a TK encoding plasmid, which was originally used to transfect the positive control cell line 143B and was purchased from ATCC. The digestion enzymes used to check the construct of this plasmid were purchased from New England BioLabs, Ipswich, MA, USA. The size of this construct was 11.3 Kb and the markers were ampR and TK+ (fig. 2.1)

Material	Supplier
E-coli strain (DH5 α)	Invirtogen life technology
QIAGEN plasmid purification minikit	QIAGEN, Germany
Agar culture medium	Sigma, Gillingham, Dorset, UK
L-broth culture medium	Central services, BLCR-UK

Ampicillin	Sigma, Gillingham, Dorset, UK
Effectene transfection reagent	QIAGEN, Germany
Colony glass rings	Bellco Glass, INC, Vineland, USA

pMLBKTK plasmid vector

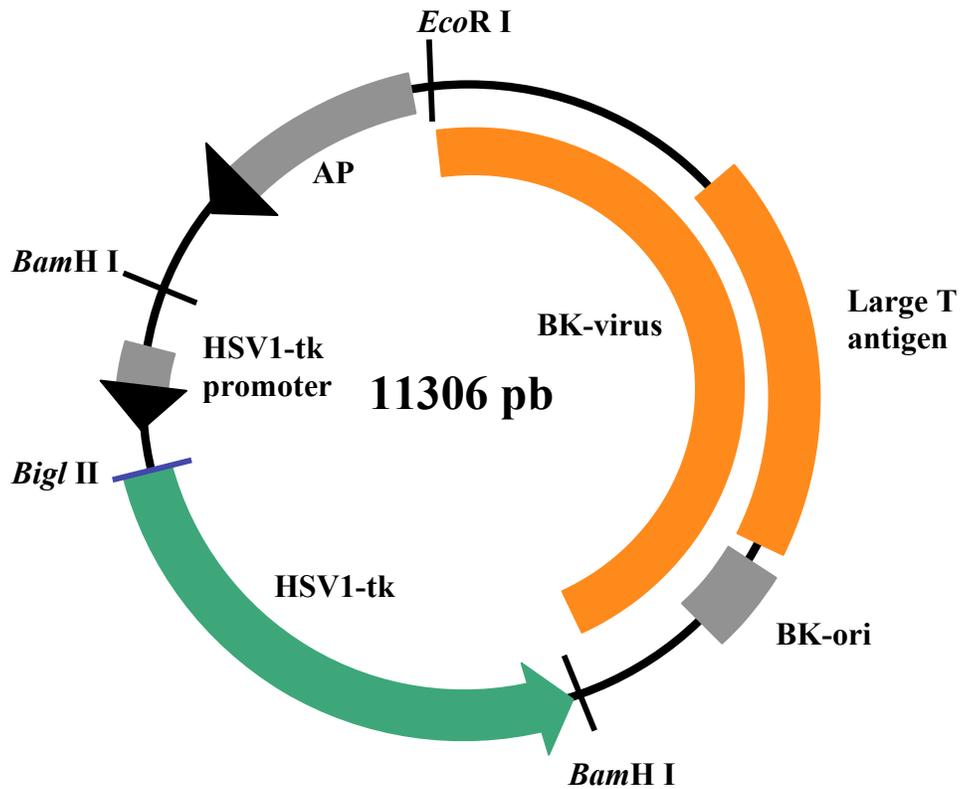


Figure 2.1: HSV1-*tk* encoding pML BK TK plasmid transfection vector.

2.1.4 Transfection efficiency

Material	Supplier
Radiolabelled FIAU ($[^{123}\text{I}]\text{FIAU}$)	Department of Radionuclide Dispensary at the Western Infirmary of Glasgow, Glasgow, UK
[Methyl- ^3H]thymidine	Amersham Biosciences UK Ltd, Buckinghamshire, UK
Ganciclovir (GCV)	Sigma, Gillingham, Dorset, UK
MTT	
Glycine	Fisher Scientific UK Ltd, Loughborough, UK
NaCl	
NaOH	
Scintillation liquid	National Diagnostic, Atlanta, Georgia, USA
TCA	Central services, BLCR-UK

2.1.5 RT-PCR

Material	Supplier
RNeasy Mini Kit (50)	Qiagen, Germany
GAP-DH Primers	
Agarose gel	Melford, Ipswich, UK
TK gene primers	

1 Kb DNA ladder	Invirtogen life technology, Paisley, UK
λ DNA/ <i>Hind</i> III ladder	
Ethidium bromide	Sigma, Gillingham, Dorset, UK
1XTBE (90 mM tris, 90 mM borate, 3 mM EDTA)	Central Services, BLCR-UK

2.1.6 Cell cycle analysis

Material	Supplier
70 % Ethanol	Fisher Scientific UK Ltd, Loughborough, UK
Propidium iodide	Invirtogen life technology, Paisley, UK
RNase	Qiagen, Germany
Cell strainer	BD Biosciences, Bedford, USA

2.1.7 Special instruments

Instrument	Supplier
Gamma counter	Packard instrument company
\square eta counter LS6000IC	
Spectrophotometer (DNA concentration) Beckman DU-64	
ELISA plate reader	Molecular Devices

2.2 Methods

2.2.1 Tissue culture

2.2.1.1 Maintenance of cell lines

The cell lines UVW, 143B and 143B-TK were grown in 90% MEM supplemented with Earle's BSS, 10% foetal bovine serum (FBS) and 2mM of L-glutamine. 143B medium was deficient in L-glutamine and supplemented with 15 μ g/ml of the selection nucleotide BUdR. 143B-TK medium was supplemented with the selection compound HAT.

DU145 cell line was maintained in 90% RPMI 1640+L-Glutamax and 10% FBS.

All culture media were kept infection free by the addition of 2.5 μ g/ml Fungizone (Amphotericin), 10,000 U/ml penicillin and 10,000 μ g/mL streptomycin.

All cell lines were tested for mycoplasma infection every two to three months. Cells were cultured in 75 cm tissue culture treated flasks (20ml medium) equilibrated with 5% CO₂ for 20 seconds and sealed tightly and incubated at 37°C. Sub-culturing into new flasks and fresh medium was carried out at each sub-confluence stage (approximately 5-8 days). Sub-confluent cultures were washed with PBS and disaggregated by treatment with 2ml of EDTA-trypsin for approximately 5 min. Fresh medium was added to neutralise trypsin and the cell suspension was pipetted up and down 4-5 times to enhance disaggregation and transferred to a universal. Cells were counted by Casey 1[®] automated cell counter (Sharfe System, Germany) and cultured at approximately 10⁵ cells per flask.

2.2.1.2 Storage of cells

Long term storage of cells was in liquid nitrogen. Cells were collected in a universal (as described above for subcultures), counted and centrifuged at 1200 rpm in 4K15 centrifuge

(Sigma) for 5 mins. Freezing medium was prepared from 80% fresh medium, 10% FBS and 10% DMSO. Cells were resuspended in the freezing medium at a density of 10^6 cells/ml and transferred to 1 ml cryotubes (Nunc vial) and placed at -70°C for 24 h to allow the cells to freeze slowly. After this time, vials were transferred to liquid nitrogen. Fresh cells were defrosted after 15 to 20 passages of the old cell lines. Cells were defrosted quickly by transferring vials from liquid nitrogen immediately to water at 37°C . Vial contents were then emptied into 75cm flasks containing fresh medium devoid of selection additives for the transfected cell lines.

2.2.2 TK plasmid construction and transfection

2.2.2.1 Introduction

2.2.2.1.1 TK Plasmid

Plasmids are extrachromosomal molecules of DNA that vary in size from 1 Kb to more than 200 Kb. Most of them are double-stranded, covalently closed, circular molecules that can be isolated from bacterial cells in superhelical form. They are found in a large variety of bacterial species. Most plasmids have a narrow host range and can be maintained in a limited set of closely related species. They are extrachromosomal elements that behave as accessory genetic units that replicate independently of the bacterial chromosome.

Plasmids have evolved a variety of mechanisms to maintain a stable copy number of plasmids in their bacterial hosts and to partition plasmid molecules accurately to daughter cells. They frequently contain genes coding for enzymes that are advantageous to the bacterial host. These genes specify a remarkably diverse set of traits, many of which are of great medical and commercial significance. Among the phenotypes conferred by plasmids are resistance to and production of antibiotics, degradation of complex organic

compounds and production of colicins, enterotoxins and restriction and modification enzymes.

pcDNA3.1(+) and pcDNA3.1(-) are vectors derived from the plasmid pcDNA3 and designed for high level stable and transient expression in mammalian hosts. High level stable and non-replicative transient transfection can be carried out in most mammalian cells. The vectors contain the following elements:

1. Human cytomegalovirus immediate-early (CMV) promoter for high-level expression in a wide range of mammalian cells.
2. Multiple cloning sites in the forward (+) and reverse (-) orientation to facilitate cloning.
3. Neomycin resistance gene for selection of stable cell lines.
4. Episomal replication in cell lines that are latently infected with SV40 or that express the SV40 large T antigen (e.g. COS-1, COS-7).

(www1.qiagen.com/Products)

2.2.2.1.2 Plasmid purification using QIAGEN plasmid Mini Kit

Transformed plasmids were purified using QIAGEN anion-exchange tips according to the manufacturer's instructions.

Briefly, with QIAGEN plasmid kits, bacterial lysates were cleared by centrifugation. The cleared lysate was then loaded onto the anion-exchange tip where plasmid DNA selectively binds under appropriate low salt and pH conditions. RNA, proteins, metabolites and other low-molecular-weight impurities were removed by a medium salt wash, and ultrapure plasmid DNA was eluted in high salt buffer. The DNA was concentrated and desalted by isopropanol precipitation and collected by centrifugation.

2.2.2.1.3 Effectene transfection reagent

Methods for transfection include calcium phosphate (Chen & Okayama, 1987; Wigler et al., 1977), lipid mediated (Felgner et al., 1987; Felgner & Ringold, 1989), and electroporation (Shigekawa & Dower, 1988).

Effectene transfection reagent is an innovative non-liposomal lipid formulation that is used in conjunction with a special DNA condensing enhancer and optimised buffer to achieve high transfection efficiencies. The enhancer first condenses the DNA molecules and effectene reagent subsequently coats them with cationic lipids, providing a particularly efficient way of transferring DNA into eukaryotic cells.

Some of the advantages using this transfection reagent are that it is fast and easy with high transfection efficiency and minimum toxicity, using small amounts of DNA.

2.2.2.1. Creating stably transfected cell lines using Geneticin selective antibiotic

The pcDNA3.1(+) and pcDNA3.1(-) vectors contain the neomycin resistant gene for selection of stable cell lines using neomycin (Geneticin). Geneticin selective antibiotic blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside, similar in structure to gentamycin and kanamycin. Expression of the bacterial aminoglycoside phosphotransferase gene (APH) derived from Tn5, in mammalian cells results in detoxification of geneticin (Southern & Berg, 1982).

2.2.2.2 TK Gene cloning

HSV1-*tk* gene was cloned into the plasmid vector pcDNA3.1 in our department. The plasmid was cleaved at the multiple cloning site (MCS) using the restriction enzymes EcoR I and Xho I and the HSV1-*tk* gene was ligated into the MCS (figure 2.2).

Plasmid construct was then checked by digestion and gel electrophoresis using a standard method.

pcDNA3.1 HSV1-TK

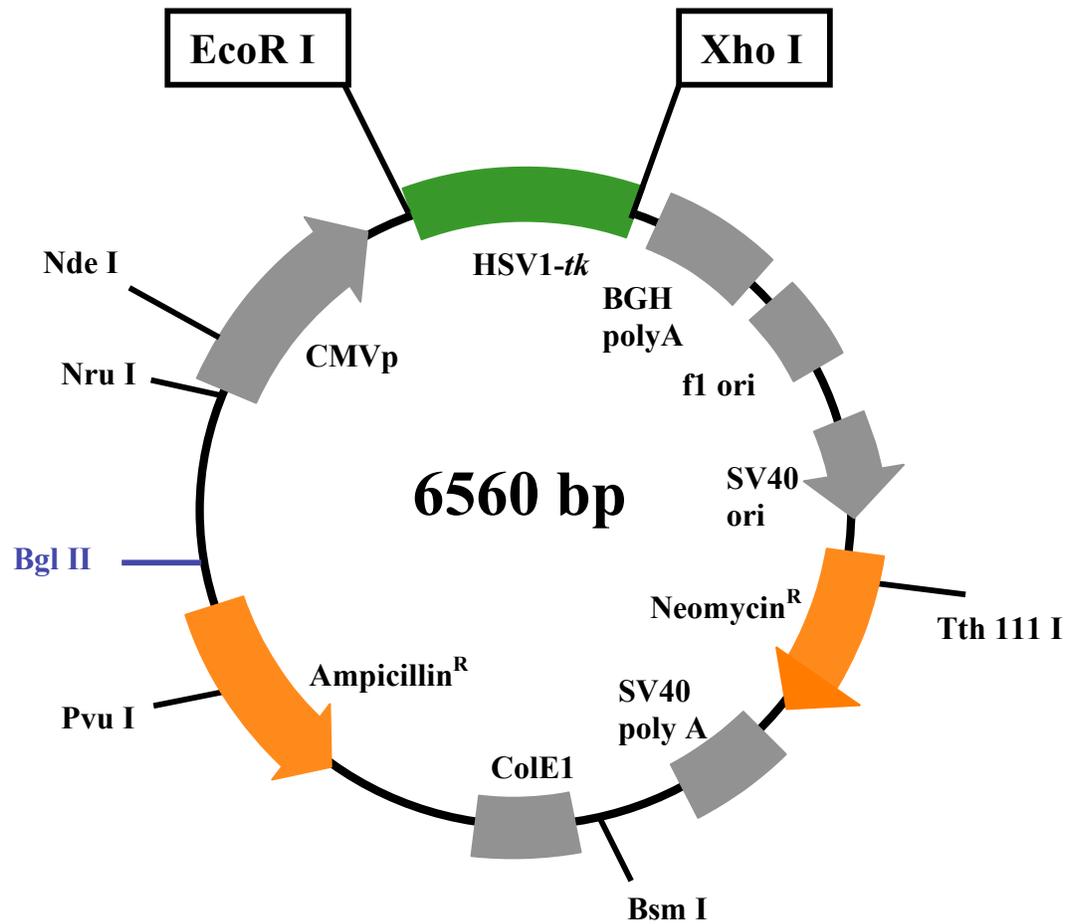


Figure 2.2: HSV1-*tk* gene was cloned in the MCS of plasmid vector pcDNA3.1 using restriction enzymes *EcoR* I and *Xho* I

2.2.2.3 TK plasmid Transformation

The TK encoding plasmids pcDNA3.1 HSV-TK and PML BK TK were propagated in the E-coli strain DH5 α . Briefly, 100 μ l of freshly thawed bacterial suspension was exposed to 1 μ g of the plasmid. After 30 min, 50 μ l of the suspension was spread on a solid agar culture medium supplemented with ampicillin (5 μ g/ml) for selection in microbiology culture plate and incubated overnight at 37°C. On the following day two discrete colonies were selected and transferred to 2 Falcon tubes containing 1ml of fresh L-broth culture medium and were incubated for 4-6 h with continuous shaking. Once culture growth was established, the entire volume was added to 500 ml of L-broth containing ampicillin and incubated overnight at 37°C with continuous shaking. Twenty-four hours later plasmids were purified utilising QIAGEN plasmid purification minikit according to the manufacturer's instructions.

2.2.2.4 TK plasmid transfection and selection

Monolayers of the cell lines DU145, PC3, U937 and 143B were transfected with pcDNA3.1TK plasmid individually using effectene transfection reagent (Qiagen, Germany) according to the manufacturer's instructions as follows:

The cells were seeded into 6-well plates at a density of 10^5 cells/ well and incubated until they reached 70-90% confluence. 1, 2 and 3 μ g of the plasmid were diluted in 150 μ l of DNA condensation buffer (EC) in 500 μ l Eppendorf tubes. 8 μ l of the Enhancer was added and mixed by vortexing for 1 second, incubated at room temperature for 2-3 min and then centrifuged for 10-15 seconds.

25 μ l of the Effectene reagent was then added to each tube, mixed by pipetting up and down and incubated at room temperature for 10 min. While waiting for the incubation period, monolayers were washed with PBS and supplied with 4 ml of fresh medium.

Each plasmid mixture was then diluted with 1 ml of fresh medium and added drop-wise to one well of the 6-well plate. The other 3 wells were used as negative controls for the

selection efficiency. The cells were then transferred to 37°C and 5% CO₂ incubators and fresh medium was replaced after 24 h.

One day later, the selection medium containing 500 µg/ml geneticin (G418) was added.

Low power microscopic examination for selection was carried out regularly and the selection medium was replaced every 3-4 days. Once complete clearance of the control wells was observed and discrete colonies were formed, several individual colonies were transferred to 25 cm² tissue culture flasks, one colony per flask, using sterile colony isolating glass rings. Selection continued in the culture flasks until cell number was enough to be transferred to the larger 75 cm² flasks to perform transfection efficiency experiments as described in the following section.

2.2.3 Transfection efficiency

2.2.3.1 GCV sensitivity (MTT assay)

The clonogenic assay is not suitable for all cell lines and not amenable to automation. Contrary to the clonogenic assay, MTT assay is quick and allows a large number of assays to be carried out in one batch (Cole, 1986). The use of tetrazolium dye (MTT) as an indirect measure of cell number was first reported in the early eighties (Mosmann, 1983). Cells in exponential phase of growth are exposed to a cytotoxic drug. The duration of exposure is usually determined as the time required for maximal damage to occur but is also influenced by the stability of the drug.

After removal of the drugs the cells are allowed to proliferate for two to three doubling times in order to distinguish between cells which remain viable and capable of proliferation and those which remain viable but cannot proliferate. Surviving cell numbers are then determined indirectly by MTT dye reduction. MTT is a yellow water soluble tetrazolium dye that is reduced in the mitochondria of living but not dead cells to a purple formazan product that is insoluble in aqueous solutions (Liu et al., 1997). The amount of

MTT-formazan produced can be determined spectrophotometrically once solubilised. The procedure was done according to an optimised assay described by J Plumb, 1999. (Plumb) The MTT dye (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) was purchased from Sigma and prepared at a concentration of 5 mg/ml in PBS. This was filter-sterilised, which also removes any reduced MTT, and can be stored at 4°C in the dark for 1 month. Glycine buffer was prepared by adding 0.1 M glycine to 0.1 M NaCl and its pH was adjusted to 10.5 using 1 M NaOH.

MTT assay was carried out in 96-well plates. Sub-confluent monolayer cultures were trypsinised and diluted to a concentration of 5×10^3 cells/ml. Cell suspension was then transferred to a Petri dish and using a multi channel pipette, 200 μ l of growth medium were added to the central 6 wells starting with column 2 and ending with column 11, 200 μ l of fresh growth medium were added to the 6 wells in column 1 and 12. Plates were incubated in a humidified atmosphere at 37°C for 2-3 days until cells are in exponential phase. Treatment was prepared in serial 4-fold dilutions of the ganciclovir (GCV) in growth medium to give eight concentrations from $10^3 \mu\text{g/ml}$ to $61 \times 10^{-3} \mu\text{g/ml}$.

Growth medium from columns 3-10 was removed and replaced with the medium containing the drug, highest concentration in column 3 and lowest concentration in column 10. Fresh medium was added only to the wells in columns 2 and 11. Plates were incubated at 37°C and 5% CO₂ for 3 days. At the end of the exposure period, medium from all wells containing cells was removed and replaced with fresh medium. Cells were left to grow for a further two days and MTT assay was performed at the fifth day after the beginning of treatment. 200 μ l of fresh medium and 50 μ l of the MTT solution (5 mg/ml in PBS) were added to all wells in column 1 to 11. Plates were wrapped in aluminium foil and incubated for 4 h in humidified atmosphere at 37°C and 5% CO₂. Medium and MTT were removed from the wells. Formazan crystals were dissolved by adding 200 μ l of DMSO to all wells in column 1 to 11 and glycine buffer 25 μ l to all wells containing

DMSO. Absorbance at 570 nm with the wells in column 1, which contains medium, MTT but no cells was used as a blank. A graph of absorbance (y axis) against drug concentration (x axis) was constructed. The mean absorbance reading from the wells in column 2 and 11 was used as a control absorbance and the IC₅₀ concentration was determined as the drug concentration required to reduce the absorbance to half of the control.

2.2.3.2 [¹²³I FIAU uptake

2.2.3.2.1 Synthesis of no-carrier-added [¹²³I]FIAU

Radio-iodinated FIAU was originally prepared by direct radio-iodination of its unsubstituted precursor, 1-(2'-fluoro-2'-deoxy-β-D-arabino-furonosyl) uracil (Misra et al., 1986) This involves heating of the precursor at 115°C with nitric acid for 90 min. the radiochemical yield ranged from 35-90% and was isotope dependent. This method has been modified by Tjuvajev (Tjuvajev et al., 1996) for the no-carrier-added (n.c.a) synthesis. Vaidyanathan & Zalutsky have developed an efficient method for the preparation of n.c.a FIAU and its astatinated analogue, FAAU, from a tin precursor (Vaidyanathan & Zalutsky, 1998).

In our study, [¹²³I]FIAU was synthesised at the Department of Radionuclide Dispensary via electrophilic iododestannylation of the corresponding trimethylstannyl precursor using peracetic acid as the oxidant following a procedure modified from the previous method described by (Vaidyanathan & Zalutsky, 1998). The final product had a radiochemical purity of > 98% (Fig. 2.3).

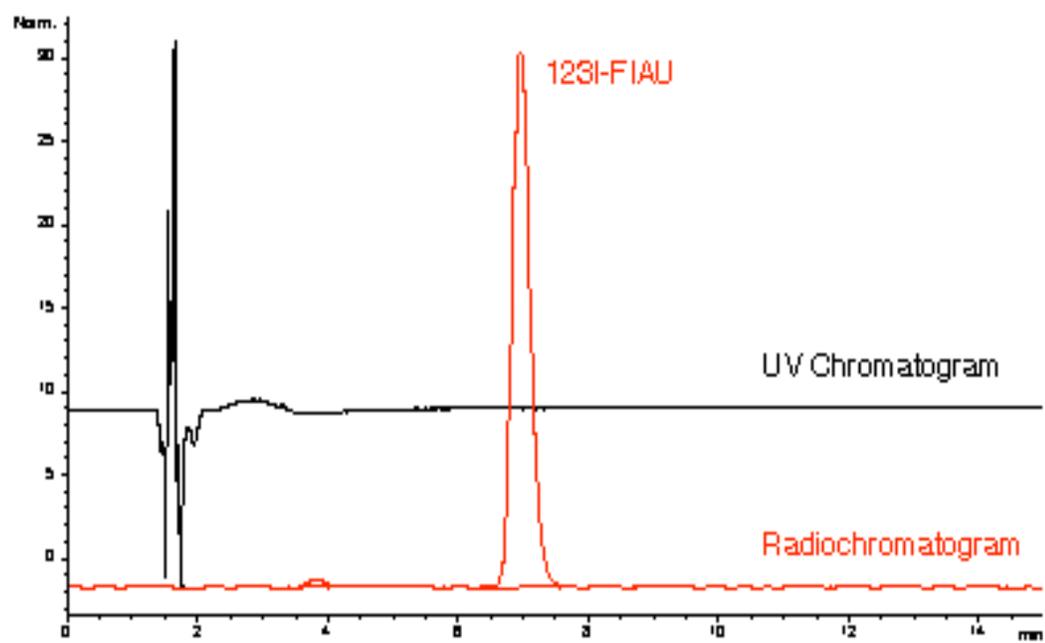
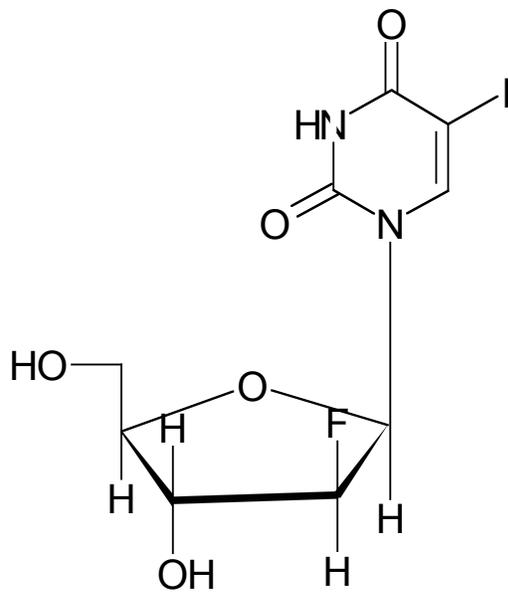


Figure 2.3: HPLC of [^{123}I]FIAU produced by no-carrier-added method showing radiochemical purity of > 98%.



5-iodo-1(2-deoxy, 2-fluoro-β-D-arabinofuranosyl)uracil (FIAU)

Figure 2.4: Chemical structure of FIAU

2.2.3.2.2 Pilot study to determine cellular uptake of [¹²³I]FIAU

[¹²³I]FIAU uptake by direct measurement with gamma-counter

In a pilot study, TK gene expression of the transfected cell lines was assessed by the direct measurement of [¹²³I]FIAU uptake compared with that of the parental cell lines.

Cells in exponential phase were seeded into 6-well plates at a density of 10⁵ cells/ well and incubated for 24 h at 37°C in 5% CO₂. Monolayers were then incubated with 7 kBq of [¹²³I]FIAU in 1ml of growth medium for a period of 2 h in triplicate for each cell line. Medium was then removed and cells were washed several times with cold PBS until no activity could be eluted. Intracellular radioactivity was then extracted by exposing cells to 1 ml of cold trichloroacetic acid (TCA) for 1 h. Extracts were transferred to 1 ml Eppendorf tubes and activity was measured in a gamma counter (Packard Instrument Company). Simultaneously, cells in untreated control plates were trypsinised and counted to determine intracellular activity relative to cell number.

This simple method obviously has a clear deficiency, it was not possible to determine non-specific uptake of [¹²³I]FIAU and its relation to cellular proliferation. Therefore, the following method of differential uptake of [¹²³I]FIAU and [methyl-³H] TdR was adopted.

2.2.3.2.3 [¹²³I]FIAU uptake normalised to [methyl-³H]thymidine uptake

This procedure was modified from the method originally described by Tjuvajev (Tjuvajev et al., 1995). The intracellular accumulation of [¹²³I]FIAU was normalised to that of [methyl-³H]TdR to account for the differences in cellular proliferation. [¹²³I]FIAU uptake was plotted against [methyl-³H]TdR uptake and data were expressed as [¹²³I]FIAU/[methyl-³H]TdR uptake ratio at the time point of maximum uptake.

Monolayers of TK+ and TK- cell lines were grown in 75 cm flasks until they reached exponential phase. Adherent cells were washed with PBS, trypsinised, counted and

transferred to 1 ml Eppendorf's tubes at a concentration of 10^5 cells/ tube in triplicates for each time point of the uptake study. Cells were kept in suspension by intermittent shaking. 1 ml of fresh medium containing 7 kBq of [123 I]FIAU and 7 kBq of [methyl- 3 H] TdR was added to each tube. Cells were incubated at 37°C and 5% CO₂. Radioactivity was counted at 10 min, 30 min, 60 min, 90 min, 2 h, 4 h and 8 h in the β and α counters as described below.

At each time point, cells were centrifuged for approximately 30 seconds. The resultant supernatant was removed carefully without disturbing the cell pellet and cells were washed at least twice with cold PBS and supernatant wash was removed by the same method to eradicate residual extracellular activity. Cell pellets were then transferred immediately to the β -counter to measure the cellular [123 I]FIAU uptake. Contamination by [methyl- 3 H]TdR radioactivity of the counts assigned to [123 I]FIAU uptake was excluded by measuring 7 kBq of [methyl- 3 H]TdR activity separately in the β -counter where no β -radioactivity was detectable. However, the radioactivity of the Auger electrons emitted by 123 I was detectable by the β counter. 123 I has a short half-life of 13 h, whereas 3 H has a half-life of 12.35 years indicating higher stability than 123 I.

Therefore, cell pellets were then stored at 4°C for at least 2 weeks to allow nearly complete decay of [123 I]FIAU. Cell pellets were then re-suspended in 1 ml PBS, added to 10 ml of scintillation fluid and transferred to the β counter to measure [methyl- 3 H] TdR cellular uptake.

2.2.3.3 RT-PCR

2.2.3.3.1 RT-PCR reaction

Transfer of the viral *tk* gene to the transfected cell lines was further assessed by reverse transcription (RT)-PCR. RNA from tumour cells was extracted using RNeasy Mini Kit (50) (Qiagen, Germany). 1 μ g of RNA was used to synthesise cDNA in 20 μ l reaction

mixtures containing 50 mM MgCl and PCR buffer by a standard protocol (GeneAmp®RNA PCR, Applied biosystems, USA) using oligo dT as a primer and Moloney-murine leukaemia virus reverse transcriptase (MML-V RT). RT cycling steps were 42 °C for 15 min, 99 °C for 5 min and 4 °C for 5 min. PCR reactions were carried out with 10 µl of RT products in 50 µl reactions using Amp *Taq* polymerase in PCR buffer containing 50 mM MgCl₂ with annealing temperature of 63°C.

Primers 5'-GGCATGCCTTATGCCGTGACCGAC and 5'-CCAGGTCGCATATCGTCGGGTATGG, were used to amplify the 709-bp *tk* specific fragment (Su et al., 1997). The housekeeping gene GAP-DH was amplified with its specific primers and served as an internal control.

2.2.3.3.2 Agarose Gel electrophoresis

The products of RT-PCR reactions and restriction enzyme digest were separated by agarose gel electrophoresis. The gel was made by melting 1.25 g of electrophoresis grade agarose in 100 ml 1XTBE (90 mM tris, 90 mM borate, 3 mM EDTA) by heating in a microwave oven. After the solution had cooled, 0.4 µg/ml of ethidium bromide was added and the gel was then poured into the electrophoresis tank. 1kb and λHINDIII ladders were used as molecular size markers. 2 µl of loading dye (15% ficoll, 0.25% bromophenol blue) was added to 10 µl of DNA and the total volume loaded into the agarose gel. Electrophoresis was carried out in a horizontal electrophoresis tank containing 1XTBE for approximately 1 h at 100 V. The separated DNA fragments were visualised by UV-transillumination of the gel.

2.2.4 Clonogenic assays for [¹²³I] FIAU sensitivity

Sub-confluent monolayers of TK+ and TK- clones of each cell line were seeded in 25 cm² flasks at 1-2 ×10⁵ cells/ flask. The cells were incubated for 48 h to reach approximately 50-70% confluence. The medium was removed; cells were washed once with PBS and

incubated with a range of doses of [¹²³I] FIAU. Cells were then incubated for 4 h, 8 h or for the total period of doubling time of each cell line at 37°C in 5% CO₂.

At the end of the treatment period, [¹²³I] FIAU containing medium was removed, cells washed with PBS until no radioactivity could be eluted (at least twice), trypsinised and seeded out for the clonogenic assay. The cells were counted using a haemocytometer and seeded into 60 mm tissue culture dishes containing 5 ml of fresh medium at a density of 1 or 3 × 10³ cells/ dish, according to each cell line's plating efficiency, in triplicate for each treatment dose. The dishes were then placed in plastic boxes and incubated at 37°C in 5% CO₂ for approximately 10-14 days until discrete colonies were observed macroscopically. The colonies were then washed with PBS, fixed gradually with 50% then 100% methanol and stained with 10% Giemsa stain or crystal violet. Colonies greater than 50 cells were counted manually and surviving fractions were calculated as a percentage of the untreated control colony number.

Calculating the surviving fraction

$$\text{Surviving fraction} = \frac{\text{Mean number of colonies of treated cell line}}{\text{Mean number of colonies of untreated control}}$$

2.2.5 Clonogenic assays for GCV sensitivity

Cells were plated cultured as described for the clonogenic assay for FIAU sensitivity. Once the cells reached an exponential phase of growth, they were treated with five to eight 4-fold dilution doses of GCV ranging from 10³ μg/ml to 61 × 10⁻³ μg/ml. At the end of the treatment period of 72 h, the cells were seeded out in 60 mm tissue culture dishes as described for the clonogenic assay for [¹²³I] FIAU sensitivity. Fixing and staining were also performed using the same method.

2.2.6 Cell cycle analysis

The cell cycle analysis was carried out for the cell lines DU145-TK and 143B-TK treated with various doses of either [¹²³I]FIAU or GCV. The cells were seeded in 75 cm flasks at a density of 10⁶ cells/ flask and cultured to reach an exponential phase of growth. Single treatment with either [¹²³I]FIAU or GCV was given as described in sections 2.2.3 and 2.2.4. At the end of the exposure period, the cells were washed with PBS, trypsinised and then collected by centrifugation at 1200 rpm for 5 min.

The cells from each treatment dose were then transferred to a universal, fixed and stained separately. The cells were fixed by drop-wise addition of cold 70% ethanol while vortexing to ensure fixation of all cells with minimal clumping. They were then kept at 4°C for at least 30 min or until staining. After removal of ethanol and washing twice with PBS, cells were recovered by centrifugation at 2000 rpm and discarding the supernatant. Cells were then stained with 500 µl of propidium iodide (50 µg/ml). They were treated with ribonuclease (50 µl of 100 µg/ml RNase) prior to staining to ensure that only DNA was stained.

Cell cycle analysis was then carried out by flow cytometry FACS Scan (Becton Dickinson) and the results were analysed by CELLQUEST program. The minimum cell number for each analysis was 10⁵ cells/ analysis.

2.2.7 Doubling time of the cells

Initially cells from exponential phase of growth of the monolayers of each cell line were seeded in 24-well plates at a density of 10⁴ cells/ well. The cells from each 3 wells were collected every 24 hours and counted separately until the growth reached plateau phase, manifested by full confluence. The growth curves were constructed using a semi-log plot and doubling times were calculated from the linear part of the curve as follows:

The general formula that describes the growth of cells:

$$A = A_0 2^n$$

Where A is the number of cells at any time, A_0 is the number of cells at an initial point, and n is the number of cell divisions that have taken place. The value n can also be described as:

$$n = T/T_c$$

Where T is the time elapsed and T_c is the doubling time of the cells. Thus the proliferation formula can be written as:

$$A = A_0 2^{T/T_c}$$

Solving for T_c , by taking the natural log of both sides, the formula becomes:

$$\ln A/A_0 = T/T_c \ln 2$$

$$2.3 \log A/A_0 = T/T_c \times 0.69$$

$$T_c = (T \times 0.69/2.3)/\log (A/A_0) = \mathbf{0.3T/\log A/A_0}$$
 (Wieder, 1999)

Where,

T = time elapsed in which the data are linear on the semi-log plot

A_0 = number of cells at initial point of the linear part of the curve

A = number of cells at the end of the linear part of the curve

2.2.7 Statistical analysis

All data were stored and analysed using Microsoft Excel program (Microsoft Windows XP professional version 2002). Results were, when applicable, reported as mean \pm standard deviation (s.d.). The statistical significance (P value) of the difference between the parental and the TK positive cell lines were evaluated by the Student's t test of two-sided distribution and two samples with equal variance. The statistical significance amongst single cell population, for example different incubation periods of the clonogenic

assay of a single cell line, was evaluated using paired two-sided distribution Student's t test.

IC₅₀ value (the drug concentration that inhibits the growth of 50% of cells) was calculated according to the simple linear regression using the formula $y = mx + b$, where, y is the estimated y value, x is the known x value, m is the slope of the curve and b is the y -intercept of the curve.

**Chapter 3 : HSV1-*tk* gene transfection and
evaluation of transfected cells**

3.1 Aims

1. To construct and propagate a plasmid encoding the HSV1-*tk* gene which can be used for *in vitro* gene delivery to the target tumour cell lines.
2. To transfect these cell lines with the constructed plasmid and then to evaluate *tk* gene expression using three different methods.
3. To determine IC₅₀ values of GCV treatment of the cell lines.

3.2 TK Plasmid construction and transformation

The *tk* gene was successfully inserted into the plasmid vector pcDNA3.1 by the method described in section 2.2.2.2. The correct size and orientation of the plasmid construct was confirmed by restriction enzyme digestion and gel electrophoresis (Fig. 3.1).

The construct of the positive control plasmid PML BK TK was also checked by the restriction enzyme digestion as shown in Fig. 3.2.

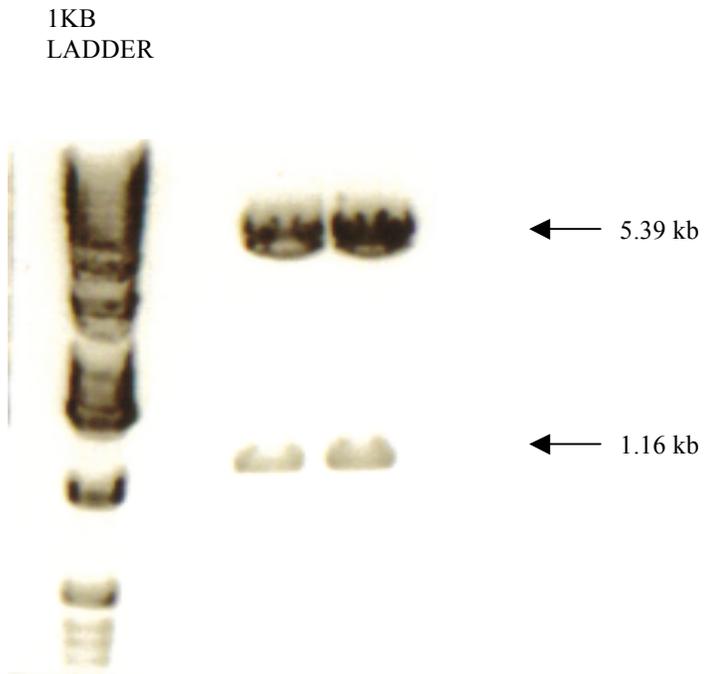


Figure 3.1: pcDNA3.1TK plasmid digest by the enzymes ECoRI and XhoI

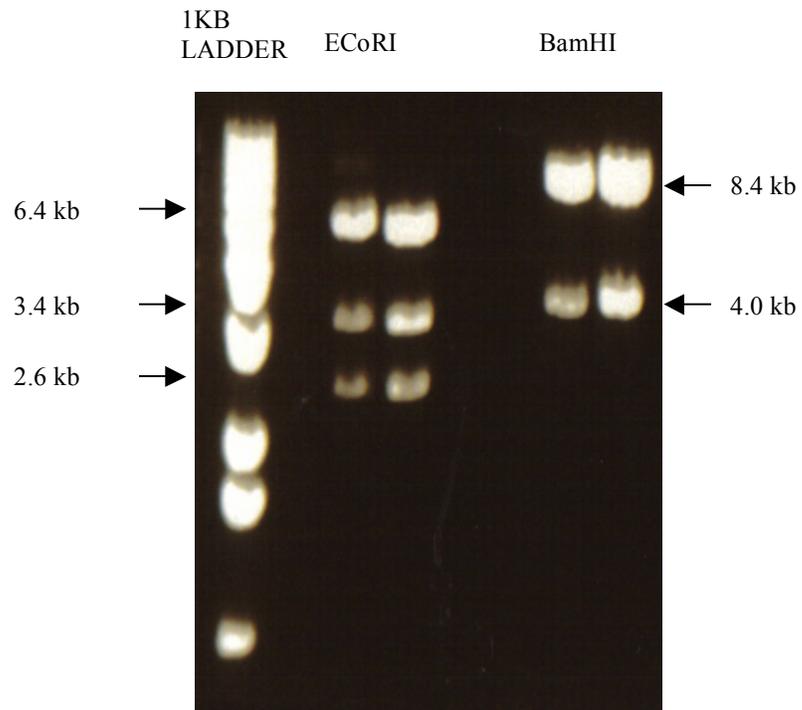


Figure 3.2: PML BK TK plasmid digestion by ECoRI and BamHI enzymes

3.3 Transfection and selection

The four cell lines DU145, PC3, UVW and 143B were successfully transfected with the plasmid pcDNA3.1TK and the expression of HSV1-TK enzyme was confirmed by the transfection efficiency experiments (section 2.2.3).

3.4 Transfection efficiency

3.4.1 Introduction

The assessment of HSV1-*tk* gene transfection efficiency *in vitro* has been described previously in several studies, e.g. (Jacobs et al., 1999; Tjuvajev et al., 1996; Tjuvajev et al., 1995). The most widely used indicators of successful transfection and subsequent expression of this gene are the increased sensitivity to acycloguanosine analogues (Tjuvajev et al., 1996; Tjuvajev et al., 1995) and the enhanced intracellular accumulation of radiolabelled thymidine analogues (Jacobs et al., 1999; Tjuvajev et al., 1996; Tjuvajev et al., 1995). Other methods include RT-PCR, Northern blot analysis (Tjuvajev et al., 1995) and Western blot of the TK protein (Jacobs et al., 1999).

In this study we have adopted three independent methods for the evaluation of transgene expression, namely, normalised uptake of [¹²³I] FIAU, GCV sensitivity and RT-PCR of the HSV1-*tk* gene.

3.4.2 [¹²³I]FIAU uptake

3.4.2.1 [¹²³I]FIAU uptake by direct measurement with gamma-counter

The monolayers of HSV1-*tk* transfected cell lines demonstrated an enhanced [¹²³I]FIAU uptake compared with the parental cell lines (Fig. 3.3). The incubation time was 2 h and the radionuclide accumulation was expressed as count per minute (CPM) per 10⁵ cells.

The enhancement factors were PC3, 3.1 (±0.4) (*P* = 0.004); DU145, 4.1(±0.5) (*P* = 0.001); UVW, 2.6(±0.34) (*P* = 0.003) and 143B, 3.8(±0.7) (*P* = 0.001)

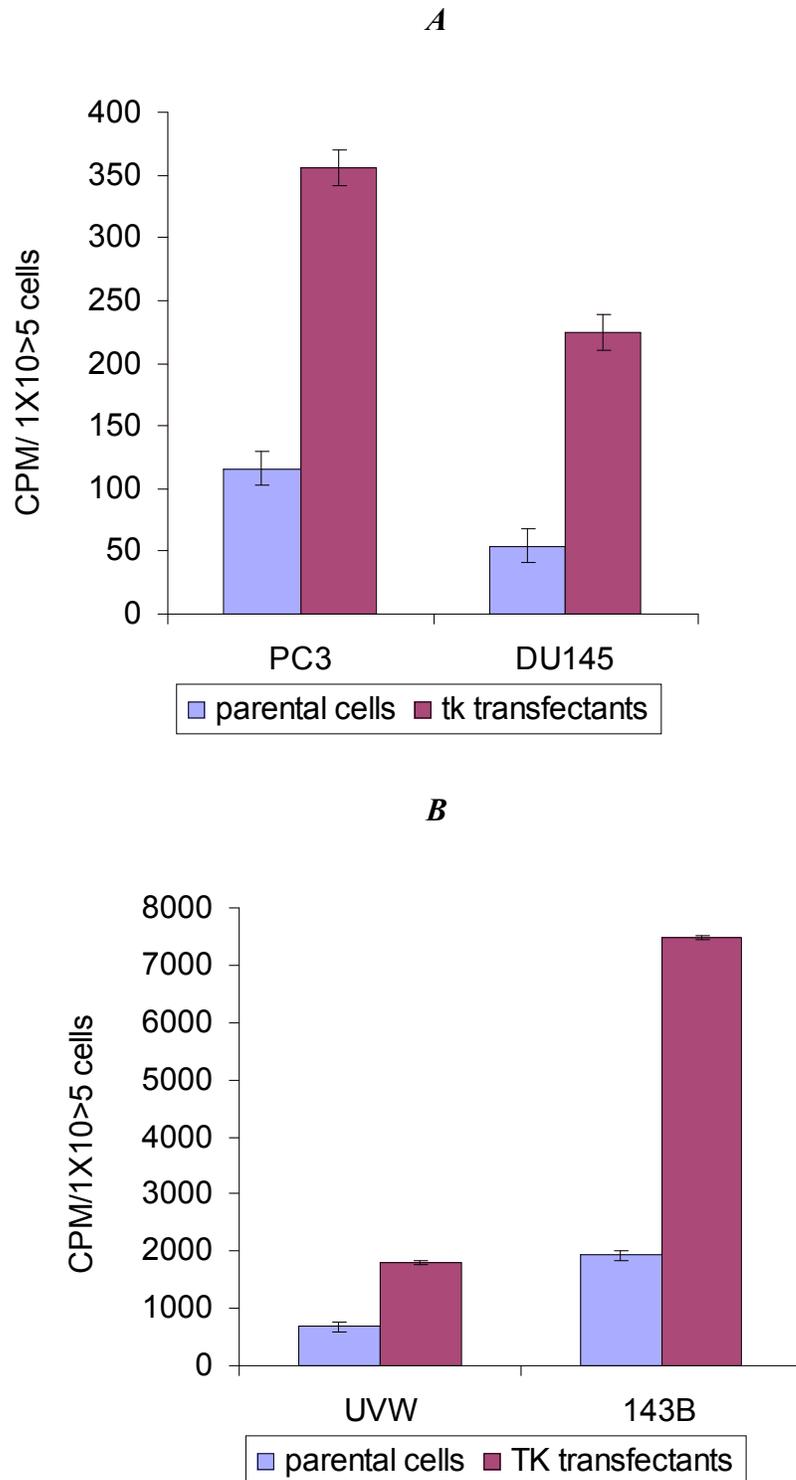


Figure 3.3: [¹²³I] FIAU uptake of Parental and TK transfectants of DU145 and PC3 (A) and UVW and 143B (B) cell lines after 2 hours incubation with 7 kBq/ ml of [¹²³I]FIAU. Direct measurement by the gamma counter indicated 2.6 to 4.1 fold increase in the uptake after TK transfection ($P < 0.005$). Data represent means and s.d. of triplicate experiments.

3.4.2.2 [¹²³I]FIAU uptake normalised to [methyl-³H] thymidine uptake

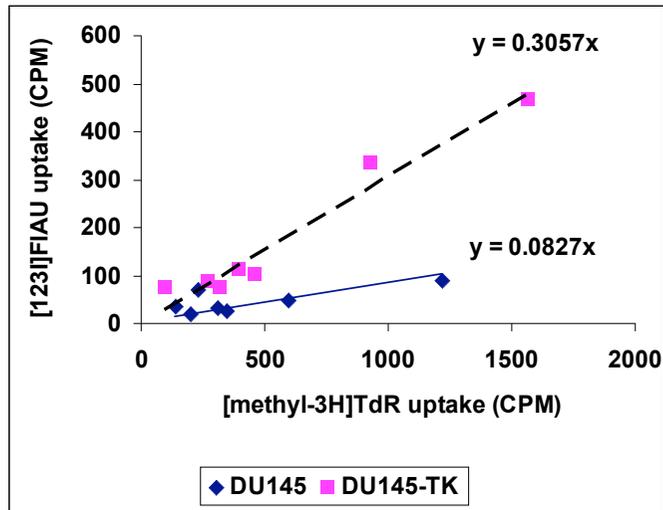
HSV-TK-positive cell lines demonstrated an enhanced uptake of [¹²³I]FIAU compared with the parental cell lines (Fig. 3.4). [¹²³I]FIAU intracellular accumulation was time-dependent and strongly correlated with cellular proliferation rate ($r = 0.976, 0.97$ and 0.91 for DU145-TK, 143B-TK and UVW-TK, respectively), which was assessed by [methyl-³H] TdR uptake. For the cell line UVW, the FIAU uptake normalised to the TdR uptake reached plateau phase after 4-8 h as indicated in Fig. 3.4C.

At the uptake study point of 8 h, [¹²³I]FIAU/ [methyl-³H] TdR uptake ratios were 4.25 (± 0.15) ($P = 0.001$), 3.59 (± 0.8) ($P = 0.01$) and 25.52 (± 3.68), times higher in the TK-transfectant than the parental cell lines of DU145, 143B, and UVW, respectively (Table 3.1). An Interesting observation was that the difference between the slopes of the normalised uptake curves of the parental and the TK positive clones of a specific cell line was close to the difference between the uptake ratios. For example, for the cell line DU145, TK transfectant slope divided by the parental cells slope was $0.3057/0.08 = 3.69$ versus 4.25 and for the cell line UVW, $1.74/0.87 = 20$ versus 25.52. This implies a similar degree of correlation between the uptake of [¹²³I]FIAU and [methyl-³H] TdR in both parental and transfected clones of each cell line.

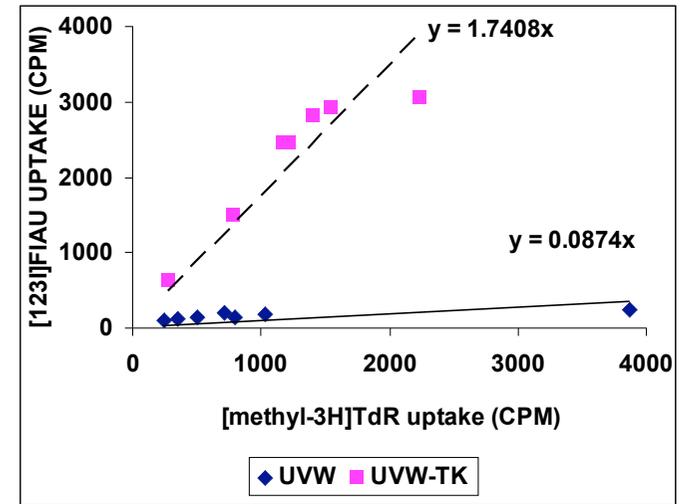
Table 3.1: FIAU/TdR uptake ratio of parental and TK-positive cell lines

	10 min	30 min	60 min	90 min	120 min	4 h	8 h
DU145	0.24	0.30	0.091	0.10	0.069	0.080	0.07
DU145-TK	0.76	0.30	0.22	0.27	0.21	0.35	0.29
143B-TK-	1.09	0.60	0.83	0.96	0.91	0.72	0.98
143B-TK	2.78	2.48	2.55	2.96	3.17	3.96	4.77
UVW	0.45	0.32	0.29	0.29	0.18	0.17	0.053
UVW-TK	2.22	1.88	1.99	2.08	2.015	1.88	1.36

A



C



B

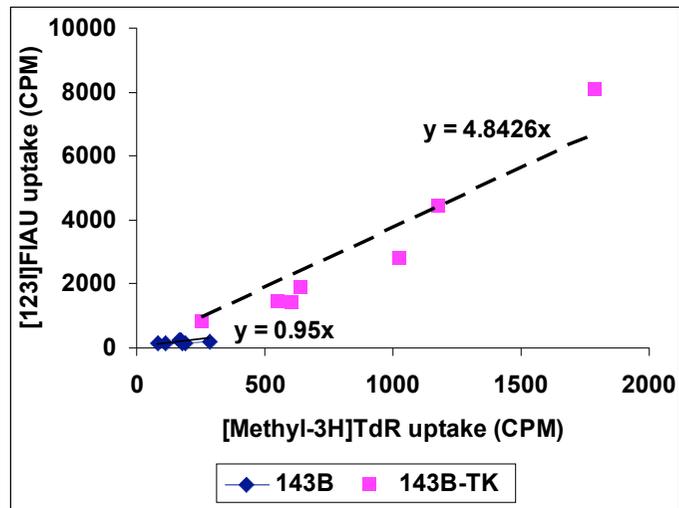


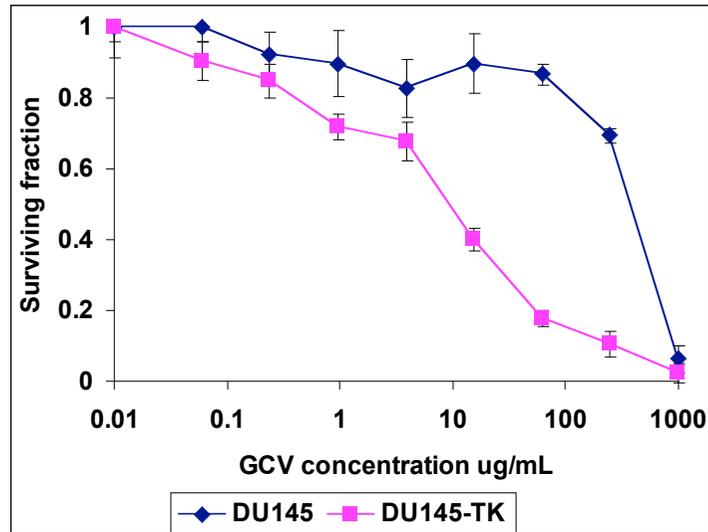
Figure 3.4: [^{123}I]FIAU uptake normalised to the uptake of [methyl- ^3H]TdR of DU1451, A; 143B, B and UVW, C. Each spot represents specific time point of the uptake study. The TK-positive clones of the three cell lines showed increasing [^{123}I]FIAU uptake which correlated to the cellular proliferation. In contrast, there was no significant [^{123}I]FIAU uptake by the parental cell lines in spite of continuous proliferation as indicated by the rising [methyl- ^3H]TdR uptake.

3.4.3 Ganciclovir Sensitivity

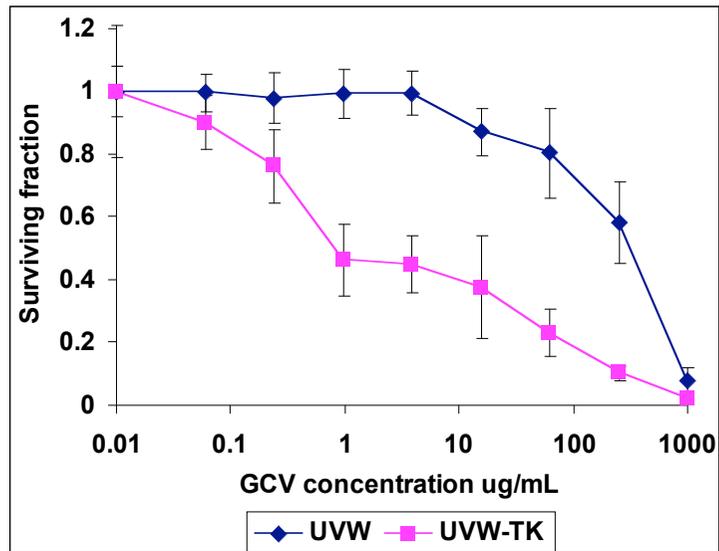
3.4.3.1 GCV sensitivity by MTT assay

There was a clear difference in GCV sensitivity between the TK-positive and the parental cell lines. 143B cells transfected by either of the two TK gene-encoding plasmids exhibited a very high sensitivity to GCV with IC_{50} approximately 3000 ($P = 0.0002$) times lower than that of the TK negative cell line (Fig. 3.5C). In the cell lines DU145 and UVW, although the sensitivities were not as high as that of the 143B cell line, they were significantly greater than those of the untransfected cell lines. The IC_{50} values were 43 ($P = 0.009$) and 212 ($P = 0.0013$) fold lower in TK positive than parental clones of DU145 and UVW, respectively (Fig. 3.5 A and B).

A



B



C

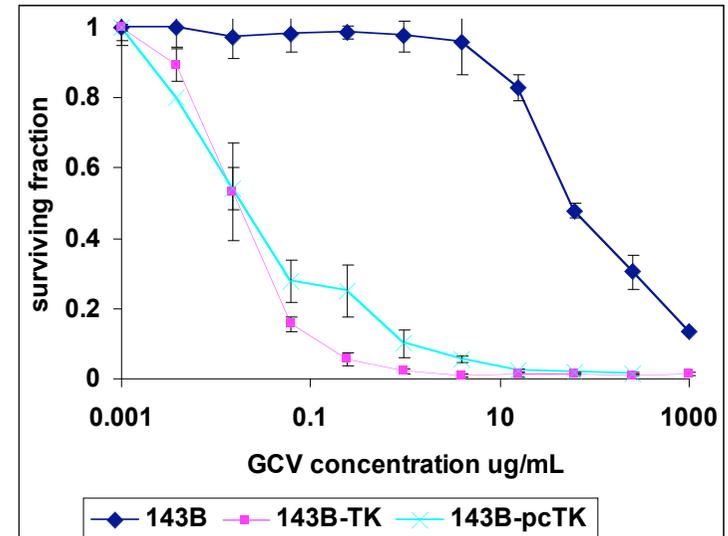


Figure 3.5: GCV sensitivity by MTT assay of the parental and TK-positive clones of the cell lines DU145, A; UVW, B and 143B, C. Enhanced GCV sensitivity of the transfected clones, compared with the parental clones, of all cell lines indicated successful HSV1-TK gene transfection. The enhancement factors (EF) of DU145, UVW and 143 were 43, 212 and 3000, respectively. Data represents mean and s.d. of triplicate experiments.

3.4.3.2 GCV sensitivity by clonogenic assay

The GCV sensitivity of the TK positive clones was further assessed by the clonogenic assay to compare GCV effect on cellular proliferation using two different assays and to calculate IC_{50} values for the subsequent experiments of combination therapy (chapter 5). It was also found that there was a discrepancy between the IC_{50} values obtained by the clonogenic assay and the MTT assay. Therefore, these two assays were compared in this section. The results showed approximately a 4-fold underestimation of GCV sensitivity in DU145 cell line using MTT assay compared with the clonogenic assays. For example, the IC_{50} of the cell line DU145-TK was 11.39 (± 0.3) $\mu\text{g/ml}$ by MTT assay and 3.2 (± 0.7) $\mu\text{g/ml}$ by clonogenic assay ($P < 0.001$) (Fig. 3.6 A). On the other hand, in the cell line 143B-TK there was a slight overestimation, where the IC_{50} was 0.025 (± 0.001) $\mu\text{g/ml}$ by MTT assay and 0.045 (± 0.01) $\mu\text{g/ml}$ by clonogenic assay ($P > 0.05$) (Fig. 3.6 B).

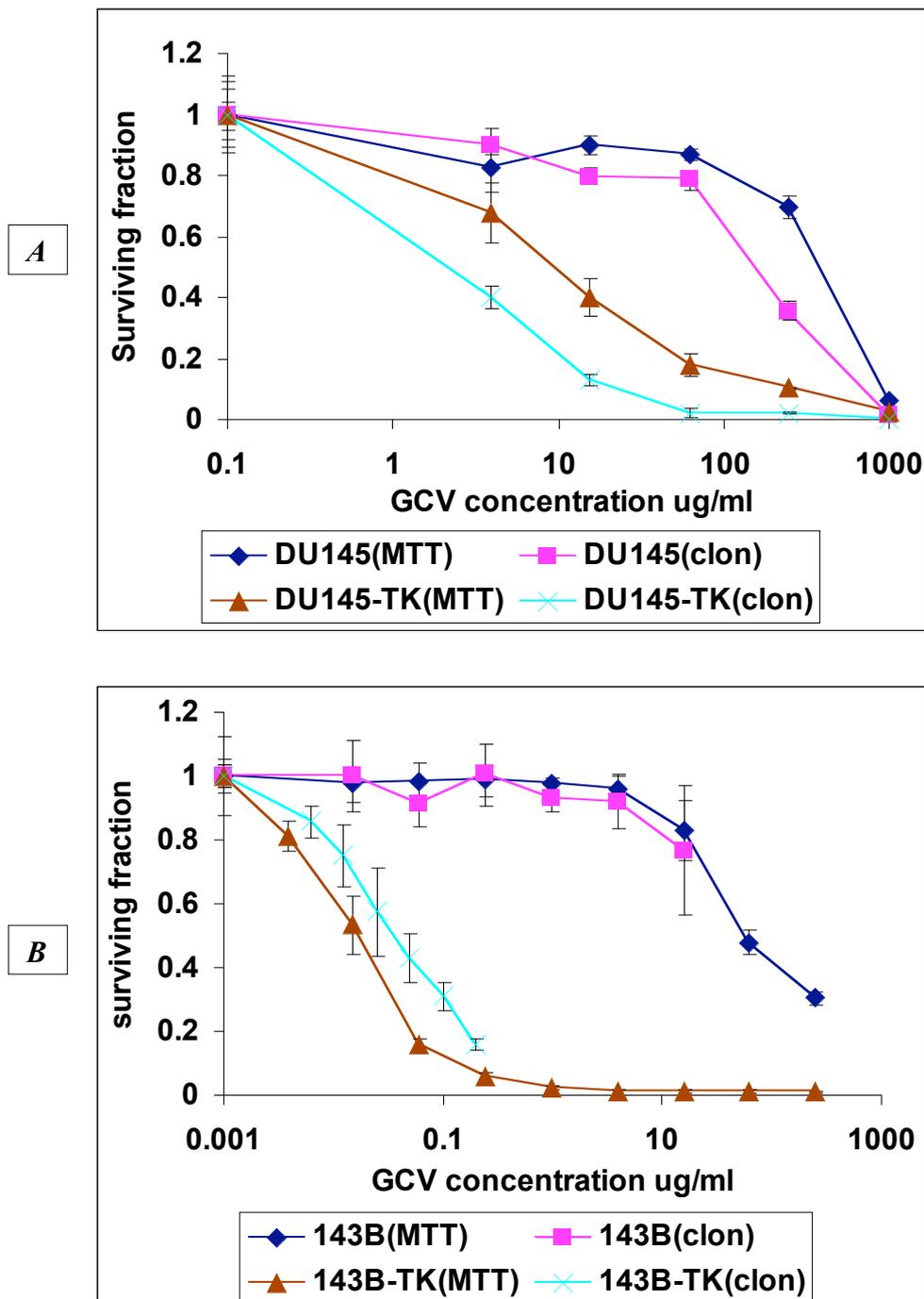


Figure 3.6: Comparison of GCV sensitivity of parental and TK positive clones of DU145 and 143B assessed by MTT and clonogenic assays. Approximately 4 fold underestimation of sensitivity by MTT assay was observed in DU145-TK cell line; *A*, and a slight overestimation was observed in 143B-TK cell line; *B*. Error bars are s.d. of 2 experiments performed in triplicates, The correlation coefficient between 2 assays of each cell line was > 0.9 .

3.4.4 RT-PCR of HSV1-TK gene

Successful transfer of the HSV1-*tk* gene to the transfected cell lines was confirmed by RT-PCR. As shown in Fig. 3.7 A, the 709 bp fragment of HSV1-*tk* gene was clearly seen in the prostate cancer cell line DU145-TK, the positive control cell line 143B-TK and was faint in the cell line UVW-TK. The cell line 143B-pcTK is the osteosarcoma cell line 143B transfected by the plasmid pc DNA3.1-TK, which has also shown a clear band of HSV1-*tk* gene.

3.5 Doubling times of the various cell lines

Growth curves of the cell lines DU143, 143B, 143B-TK and UVW are shown in Fig. 3.8 and the calculated doubling times are in Table 3.2. We note that there was a difference between the doubling time of the parental cell line 143B and that of the TK-positive cell line 143B-TK. This was attributed to the fact that the parental cell line 143B is TK-negative and therefore it has a different rate of DNA synthesis and as a consequence a longer cell cycle.

Table 3.2: The doubling times of various cell lines. Values are means and s.d. of triplicate experiments.

Cell line	Doubling time (hours)
DU145	26.22 (\pm 1.06)
143B	29.86 (\pm 3.03)
143B-TK	20.52 (\pm 2.16)
UVW	26.29 (\pm 2.64)

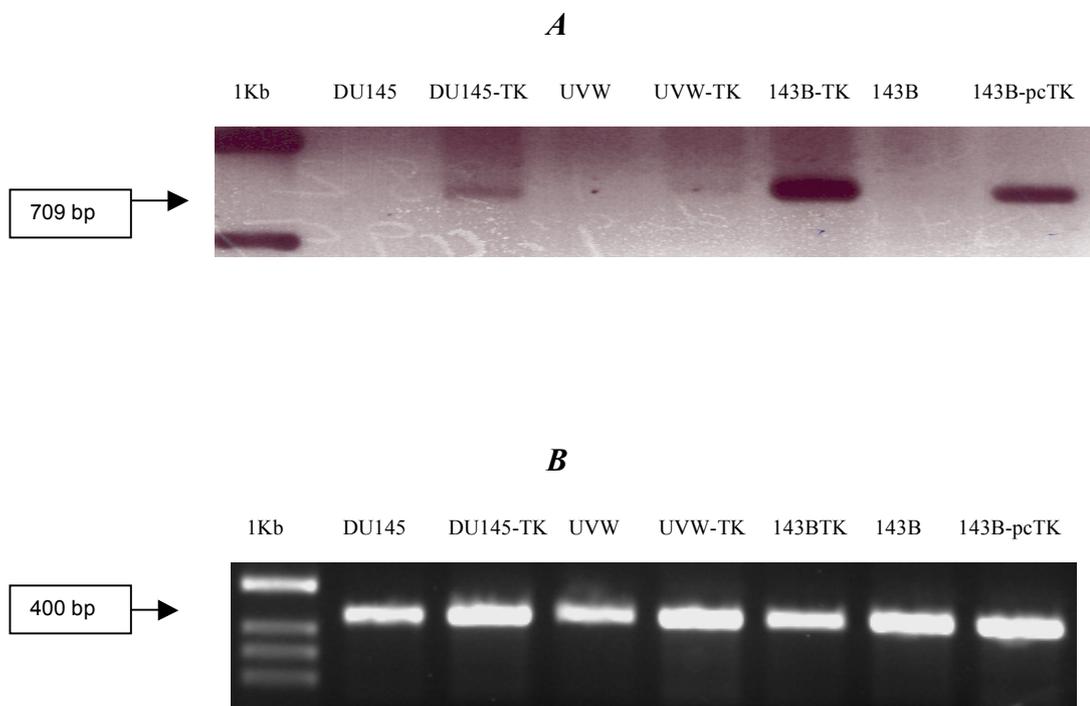


Figure 3.7: RT-PCR products for HSV1-tk gene (A) and the reference gene of GAP-DH (B) of parental and transfected cell lines. 709bp and 400bp fragments of HSV1-tk and GAP-DH, respectively, were recognised using 1kb DNA ladder (1st column from left)

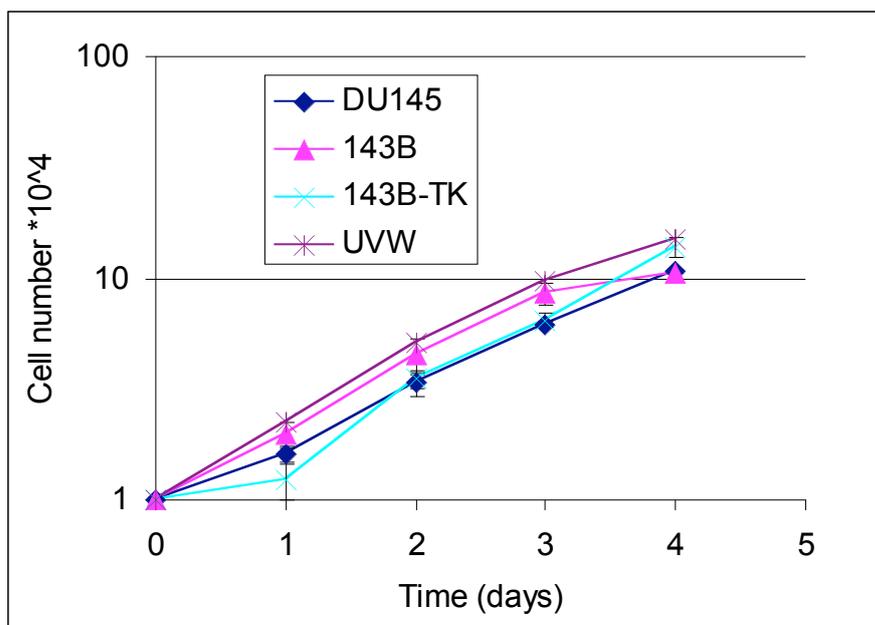


Figure 3.8: Growth curves of the cell lines DU145, 143B, 143B-TK and UVW. Data represents mean and s.d. of triplicate experiments.

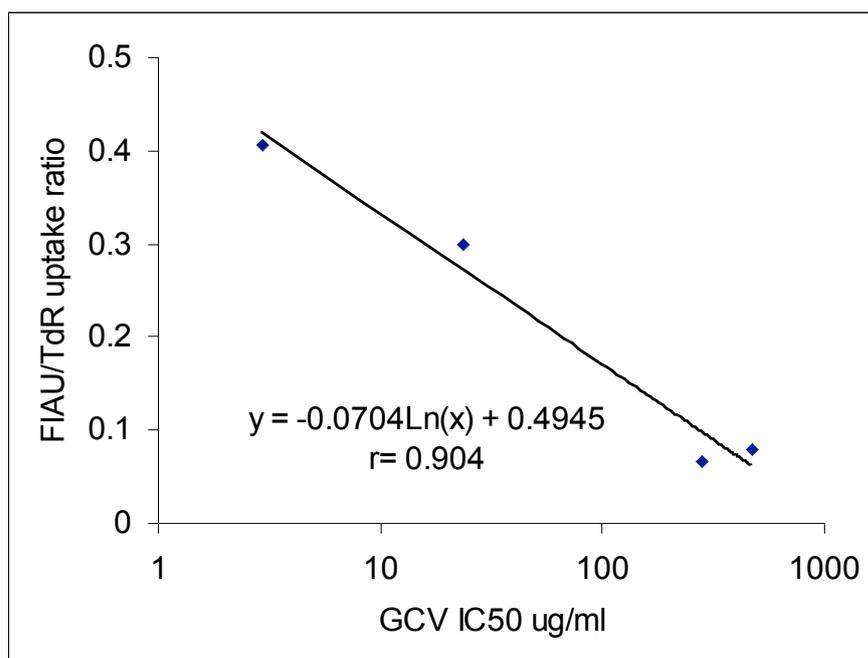


Figure 3.9: The relationship between FIAU accumulation ([¹²⁵I]FIAU / [*methyl*-³H]TdR uptake ratio) and IC₅₀ values of GCV in the different clones of TK positive cell lines. Strong correlation was observed with $r = -0.904$.

3.5 DISCUSSION

In this study, the first sign of successful transfection of the different cell lines with the TK encoding plasmids was the continuous growth of transfected cells in the selection medium. For cells transfected with plasmid pcDNA3.1-TK, this indicated co-expression of the Geneticin resistance gene and for cells transfected with PML BK TK plasmid, i.e. 143B-TK, this indicated co-expression of the HGPRT enzyme gene.

The direct measurement of [¹²³I]FIAU cellular uptake was chosen as an easy and quick method to confirm TK gene transfection. This method showed higher [¹²³I]FIAU uptake in TK positive cell lines compared with the parental cell lines. However, this method does not assess the relationship between the accumulation rate and the cellular growth rate. Furthermore, the uptake enhancement shown was less than that predicted from the GCV sensitivity enhancement by TK gene expression..

The method of normalised uptake of [¹²³I]FIAU was modified from the one described by Tjuvajev et al (Tjuvajev et al., 1995). Tjuvajev et al. studied the accumulation of the potential HSV1-TK enzyme radiolabelled substrates: IUdR, GCV and FIAU in relation to that of radiolabelled thymidine (*methyl*-[³H]TdR or 2-[¹⁴C]) to control for cell proliferation. Double label studies were performed using a beta spectrometer and standard ³H and ¹⁴C dual channel counting technique. FIAU and IUdR assays were performed after various incubation periods of 10, 30, 60, 90 and 120 min. The data were expressed as harvested cells: medium concentration ratio. This study showed low accumulation by a TK-positive glioma cell line of GCV compared to that of FIAU and IUdR and higher but not TK specific accumulation of IUdR compared to that of FIAU.

In our study, the FIAU was labelled with ¹²³I which is an Auger electron emitter measurable by gamma counting. Given the ultra-short half-life of ¹²³I (13 h) and the

long half-life of ^3H , it was necessary to measure FIAU cellular uptake immediately for the various incubation periods. Samples were then stored in 4°C for at least 2 weeks to allow for complete decay of ^{123}I and *methyl*- ^3H TdR accumulation was measured by liquid scintillation counting.

The [^{123}I]FIAU uptake by TK positive clones was found to be time and proliferation dependent and was rising up to 8 hours of incubation period (Fig. 3.4). The final data were expressed as [^{123}I]FIAU/ *methyl*- ^3H TdR uptake ratio (Table 3.1), which was significantly higher in HSV1-*tk* transfected clones than in the parental cell lines, indicating *tk* gene expression.

The second method to confirm HSV1-*tk* gene expression was by assessing GCV sensitivity by MTT assay. The MTT assay was selected because it is quick and allows large number of assays to be carried out in one batch especially if the range of effective doses for several clones is unknown. GCV has been used in clinical gene therapy protocols for treatment of tumours transduced with HSV-*tk* gene (1994).

In this study, significant enhancement of GCV sensitivity was noticed in the various cell lines after HSV-*tk* gene transfer. However, the response was variable (Fig. 3.5). The most sensitive cell line was 143B-TK. When the control cell line 143B was transfected with the plasmid pcDNA3.1TK, it demonstrated GCV sensitivity similar to the positive control 143B-TK, as shown in Fig. 3.5C. This indicated that the difference in GCV sensitivity amongst different cell lines, despite expressing similar gene, was due to the difference in cell lines' response rather than the expressed plasmid. This variation has been described years ago where the antiherpetic activity of acyclovir (ACV) was shown to be dependent upon the virus, cell type, exogenous drug concentration, time of exposure to AVC, and the presence of other nucleosides in the surrounding medium (Elion et al., 1977; Furman et al., 1981).

Furthermore, a highly significant relationship was observed between independent measures of HSV1-*tk* gene expression, namely GCV sensitivity and the level of [¹²³I]FIAU accumulation in different cell populations (Fig. 3.8). This was also observed in a previous study (Tjuvajev et al., 1995), where there was a linear relationship between mRNA and GCV sensitivity on the one hand and the level of FIAU accumulation on the other hand.

Figure 3.6A shows an underestimation of GCV sensitivity of the DU145 cell line using MTT assay in contrast to clonogenic assay. This could be attributed to the fact that MTT is performed at day 5 of treatment, following less than 4 doubling times, while the colonies in the clonogenic assay are usually counted approximately 10 days after treatment. This means that the MTT assay included all viable cells from which a proportion will die during subsequent cell divisions and fail to form colonies.

Chen et al. reported that clonogenic assay is more sensitive than the MTT assay. The ID₅₀ values (dose which inhibits 50% of cell population) obtained from the MTT assay were approximately 3 to 5 times lower than those from the other two methods (Chen et al., 1990).

Sobottka and Berger compared MTT and cell counting efficiency to assess antineoplastic agents and concluded that depending on the antitumour agent used, the MTT assay can result in slight or even considerable underestimation of the antitumour efficacy of certain compounds and may need correction by consideration of the effect of the drugs on cell size (Sobottka & Berger, 1992). However, other authors reported a high degree of correlation (up to $r = 0.939$, $P < 0.01$) (Kawada et al., 2002) between MTT and clonogenic assays results (Carmichael et al., 1987; Perez et al., 1993). Although we noted a similar degree of correlation, all subsequent sensitivity assays were carried out using clonogenic assays for several reasons.

Since it was first described (Hamburger & Salmon, 1977; Salmon et al., 1978), tumour-colony assay has been the gold-standard for assessing *in vitro* tumour sensitivity to chemotherapy agents, external beam and targeted-particle radiotherapy. It reflects the cytocidal effect of therapy rather than the possibly transient cytostatic effect that is provided by MTT assay. Another advantage is the reduced risk of radiation contamination associated with the clonogenic as opposed to the MTT assay when investigating response to targeted-particle radiotherapy.

In summary, in this chapter we have transfected 3 tumour cell lines, DU145, 143B and UVW, with a plasmid encoding the gene of HSV-TK enzyme. HSV-*tk* gene expression was then confirmed by three transfection efficiency methods.

**Chapter 4 : [¹²³I]FIAU toxicity to tumour cells
expressing the HSV1-*tk* gene**

4.1 Aims

1. To test the hypothesis that radiolabelled FIAU can be used as a targeted radiotherapy agent for tumours expressing HSV1-tk gene.
2. To explore the response of tumour spheroids to this new strategy and to assess the possibility of enhanced response by bystander or cross-fire effects.
3. To investigate the effect of serum concentration and cellular proliferation rate on the response of parental and TK-positive tumour cells to radiolabelled FIAU.
4. To determine the effect of cell cycle phase on TK-positive tumour cells treated with [¹²³I]FIAU for two different incubation periods.

4.2 Introduction

The rationale for using radiolabelled FIAU as targeted radiotherapy agent was built on two main foundations. Firstly previous experience with another thymidine analogue 5-iodo-2'-deoxyuridine (IUdR) (Fig. 4.1), indicated that it can incorporate into the DNA of rapidly dividing cells. When IUdR was labelled with Auger electron-emitter radioiodine ¹²³I or ¹²⁵I, it exerted a lethal effect on cells undergoing DNA synthesis, as shown in several studies (Hofer & Smith, 1975; Makrigiorgos et al., 1989; Schneiderman & Schneiderman, 1996).

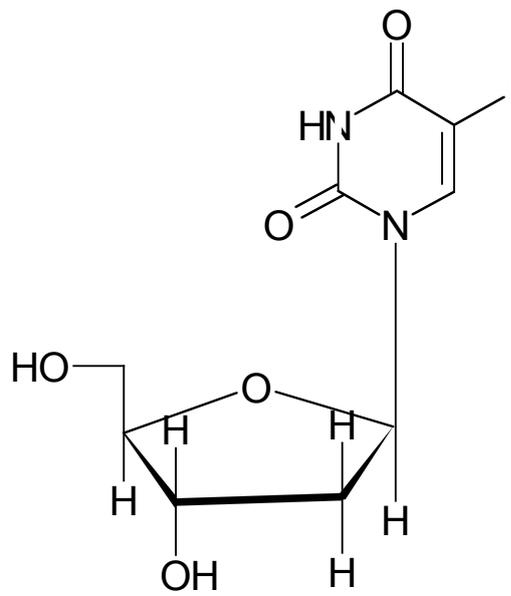
Secondly, tumour cells expressing viral TK gene can selectively accumulate FIAU by virtue of viral TK phosphorylation that could result in significant incorporation into the cellular DNA. FIAU was shown to be a substantially better substrate for the HSV1-TK enzyme than IUdR or GCV (Tjuvajev et al., 1995). Similar to IUdR, if FIAU is radio-

labelled with an Auger electron-emitter, enhanced cell kill will occur by virtue of Auger electron radiation damage. The Auger electron has high ionising radiation and ultra-short LET with effective range of only few nanometers (Martin & Haseltine, 1981). This will ensure delivering intense and localised radiation damage to the targeted tumour cells. However, in order for the Auger electron-emitters to kill cells, they must be incorporated into the cellular DNA (Kassis et al., 1987).

It has been shown that the plasma half life of FIAU in human is about 4 h (Feinberg et al., 1985; Haubner et al., 2000; King, 1991) and hence the first exposure period of tumour cells was determined according to this to mimic *in vivo* kinetics. The second period of 8 h is double the plasma half-life. This interval was selected to assess the relationship between the exposure period and sensitivity.

Both FIAU incorporation into cellular DNA and the radio-sensitivity of tumour cells depend on the phase of cell cycle. Mammalian TK has two isozymes: a minor mitochondrial isozyme which does not fluctuate during the cell cycle and the cellular isozyme which reaches a high level in cycling cells (Adler & mCauslan, 1974). TK activity and hence FIAU phosphorylation increases at or near the G1-S phases (Coppock & Pardee, 1985). Dividing cells are most radiosensitive during mitosis (M phase) and late G₂ phase of the cell cycle (Sinclair & Morton, 1966; Terasima & L.J., 1963).

If during this *in vitro* evaluation of [¹²³I]FIAU cytotoxicity all treated tumour cells are in exponential phase, i.e. all cells are cycling, an exposure period equal to their doubling time will theoretically ensure complete cell kill. Therefore, we have also evaluated tumour cell sensitivity to [¹²³I]FIAU after an exposure period similar to their doubling time. Furthermore, cell cycle analysis was carried out to assess the effect of [¹²³I]FIAU treatment on the cell cycle progress.



5-iodo-2'-deoxyuridine (IUdR)

Figure 4.1: chemical structure of IUdR

4.3 Materials and methods

4.3.1 Cell lines

The parental clones and clones expressing HSV1-*tk* gene of three different cell lines were used in these experiments. These three cell lines, DU145, 143B and UVW are able to grow both in monolayers and multicellular spheroids and their capacity to express the HSV1-*tk* gene was confirmed (see sections 3.4).

4.3.2 [¹²³I]FIAU sensitivity of monolayers

The *in vitro* sensitivity to [¹²³I]FIAU of the monolayers of parental and TK positive clones of three different tumour cell lines was determined by clonogenic assay as described in section 2.2.4. Monolayers of the three cell lines were treated with a range of concentrations of [¹²³I]FIAU for a period of 4 h, 8 h, and for a period equal to the total doubling time of each cell line.

The transfected cell lines were also treated with equivalent molar concentrations of unlabelled FIAU to determine whether any of the kill was the result of DNA incorporation of the unlabelled FIAU. The incubation period for this experiment was identical to the doubling time of each cell line.

4.3.3 [¹²³I]FIAU Sensitivity of multicellular spheroids

To investigate the possibility of cross-fire effect of [¹²³I]FIAU on a three dimensional model, multicellular spheroids were prepared and treated as follows:

Cells in exponential phase were disaggregated into a single cell suspension and counted as described previously (section 2.2.1.1). 1, 2 or 3 x 10⁶ cells were transferred to a 750 ml spinner flask containing 75 ml of fresh medium, equilibrated with 5% CO₂ and incubated at 37°C. Spheroids formed with continuous spinning for approximately 4 to 5

days and the pretreatment size of the spheroids was determined by measuring two perpendicular diameters, using an inverted phase-contrast microscope connected to an image analyser.

Spheroids with average diameter of 200 μ m were split between number of universals according to the number of doses, dosed and incubated for the determined exposure period with continuous spinning. The degree of [¹²³I]FIAU uptake by these spheroids was not determined in this experiment. The spheroids were then washed twice with PBS and once with fresh medium, trypsinised for 10 min and disaggregated thoroughly by passing them up and down a 19G needle 4-5 times. Single cell suspension was confirmed microscopically and then counted using a haemocytometer. Spheroid sensitivity was then assessed by clonogenic assay as described in section 2.2.4.

4.3.4 Cell cycle analysis

The effect of [¹²³I]FIAU exposure on cell cycle phase distribution was assessed by the method described in section 2.2.5.

4.4 Results

4.4.1 [¹²³I]FIAU sensitivity of monolayers

The clonogenic survival curves of the parental and transfected clones treated with [¹²³I]FIAU for 4 h are shown in Fig. 4.2. The three cell lines demonstrated enhanced sensitivity to [¹²³I]FIAU when transfected with the *tk* gene. The data were fitted to a curve and the IC₅₀ values calculated from the curve were significantly reduced in the TK-positive cell clones compared with the parental cells as shown in Table 4.1. The [¹²³I]FIAU sensitivity enhancement factor by TK transfection (EF) was calculated as the IC₅₀ of the parental clone divided by the IC₅₀ of the TK transfected clone of the same cell line. The EF for the cell lines DU145 and UVW were 5.2 and 2.8, respectively ($P < 0.005$).

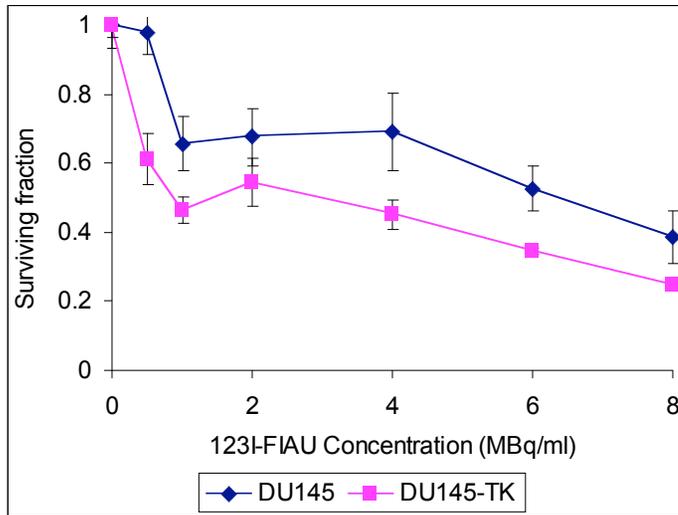
Doubling the treatment period from 4 h to 8 h further enhanced [¹²³I]FIAU sensitivity of the TK positive clones of DU145 and 143B as shown in Fig. 4.3 and Table 4.2. The IC₅₀ of the cell line DU145 was reduced from 0.8 (± 0.05) MBq/ml to 0.16 MBq/ml and the EF increased to 13.8. For the cell line 143B, it was not possible to determine IC₅₀ of the parental cell line and hence similarly the EF. For the TK positive clone 143B-TK, the IC₅₀ was reduced from 1.8 (± 0.5) MBq/ml to 0.22 (± 0.028) MBq/ml.

Table 4.1: The IC₅₀ values (MBq/ml) and EF of the the parental and TK-positive clones of cell lines treated with [¹²³I]FIAU for 4 h. Values in parenthesis are s.d. of triplicate experiments.

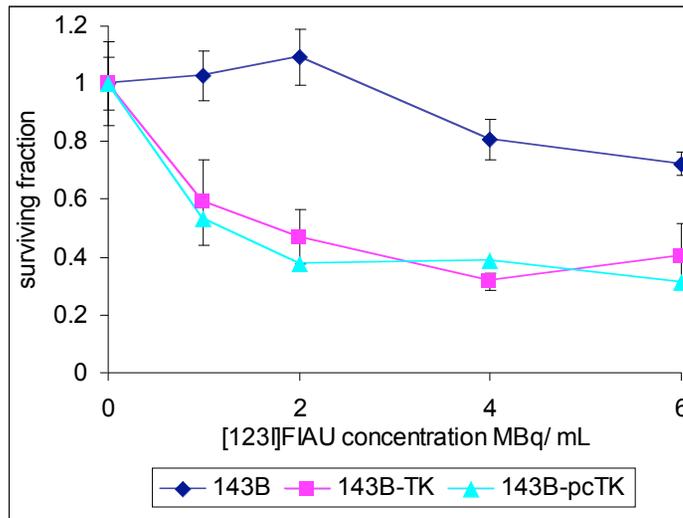
Cell line	IC ₅₀ (s.d.)	Sensitivity enhancement factor (EF) by TK	<i>P</i> value (between parental and TK-positive clones)
DU145	4.65 (0.67)		
DU145-TK	0.89 (0.05)	5.2	< 0.001
143B	ND		
143B-TK	1.8 (0.5)	ND	
143B-pcTK	2.6 (0.16)	ND	
UVW	7.03 (0.61)		
UVW-TK	2.46 (0.13)	2.8	<0.0001

ND, not determined

A



B



C

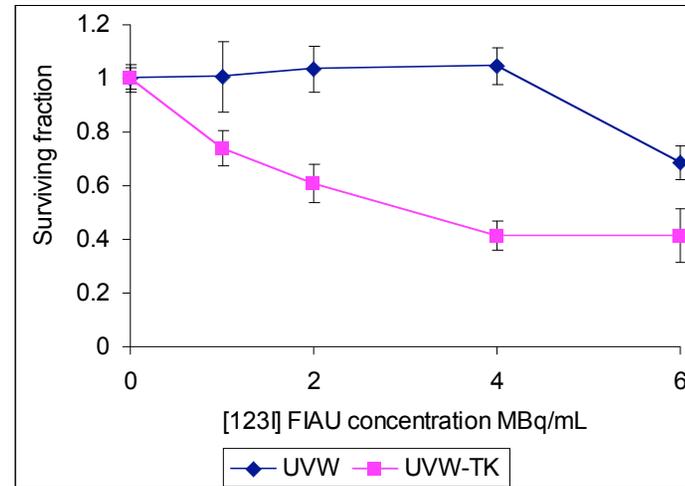


Figure 4.2: Clonogenic survival for [¹²³I]FIAU sensitivity of parental and TK positive clones of DU145, A; 143B, B and UVW, C. Monolayers were treated with increasing doses of [¹²³I]FIAU for 4 h. Data represents means and s.d. of triplicate experiments.

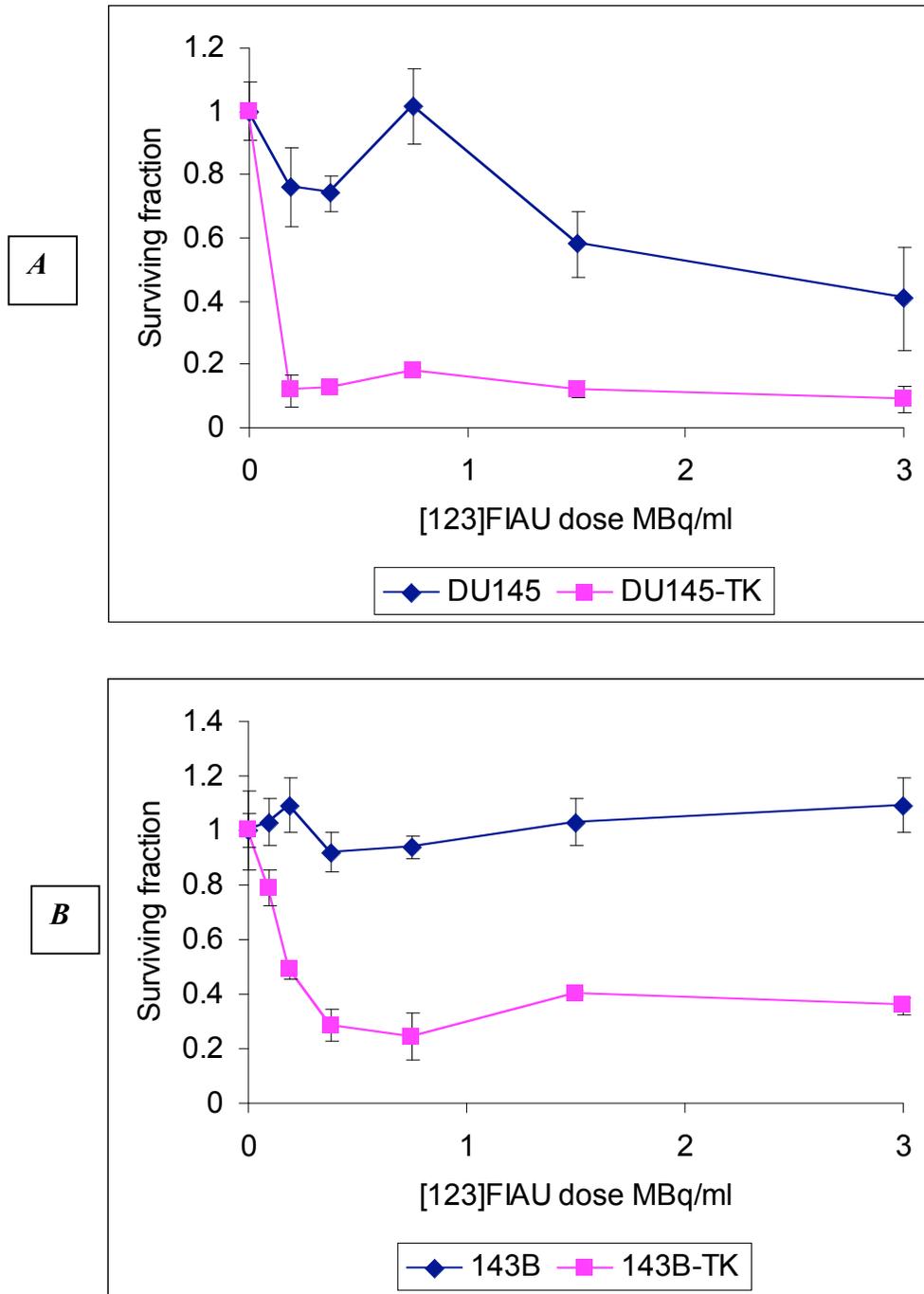


Figure 4.3: Clonogenic survival of parental and TK positive clones of the cell lines DU145, *A* and 143B, *B* treated with [¹²³I]FIAU for 8 hours.

Table 4.2: The IC₅₀ values (MBq/ ml) and EF of the parental and TK-positive clones of the cell lines DU145 and 143B-TK treated with [¹²³I]FIAU for 8 h.

	IC ₅₀ (s.d)	EF	P value
DU145	2.22 (0.11)		
DU145TK	0.16 (0.005)	13.8	0.001
143B	ND		
143B-TK	0.229 (0.028)	ND	ND

ND, not determined

4.4.3 The effect of cellular proliferation rate on [¹²³I]FIAU sensitivity

There was a strong correlation between the clonogenic survival of the tumour cell lines treated with [¹²³I]FIAU and the degree of cellular proliferation. Fully confluent (plateau phase) monolayers of the cell lines DU145-TK and 143B-TK exhibited lower sensitivity to increasing concentrations of [¹²³I]FIAU compared with the monolayers in exponential phase (Fig. 4.4). For comparison purpose, IC₂₅ values ([¹²³I]FIAU concentration that inhibits the growth of 25% of cells) were determined from the curves. The IC₂₅ of DU145-TK was 1.3 (±0.3) MBq/ml for plateau phase versus 0.4 (±0.009) MBq/ml for exponential phase ($P = 0.006$), and of 143B-TK was 3.9 (±0.4) MBq/ml for plateau phase versus 0.53 (±0.14) MBq/ml for exponential phase ($P < 0.005$).

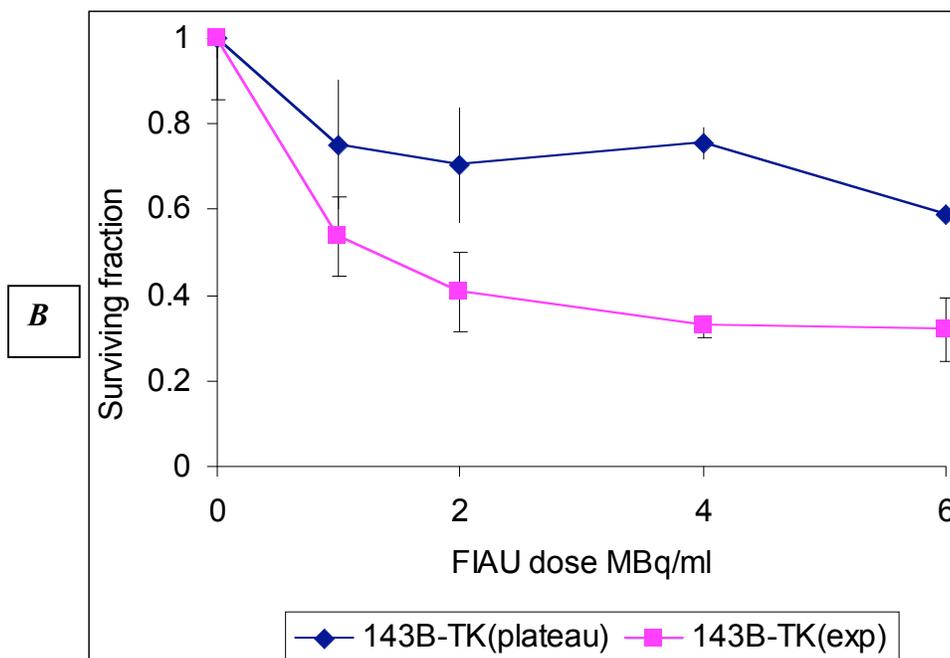
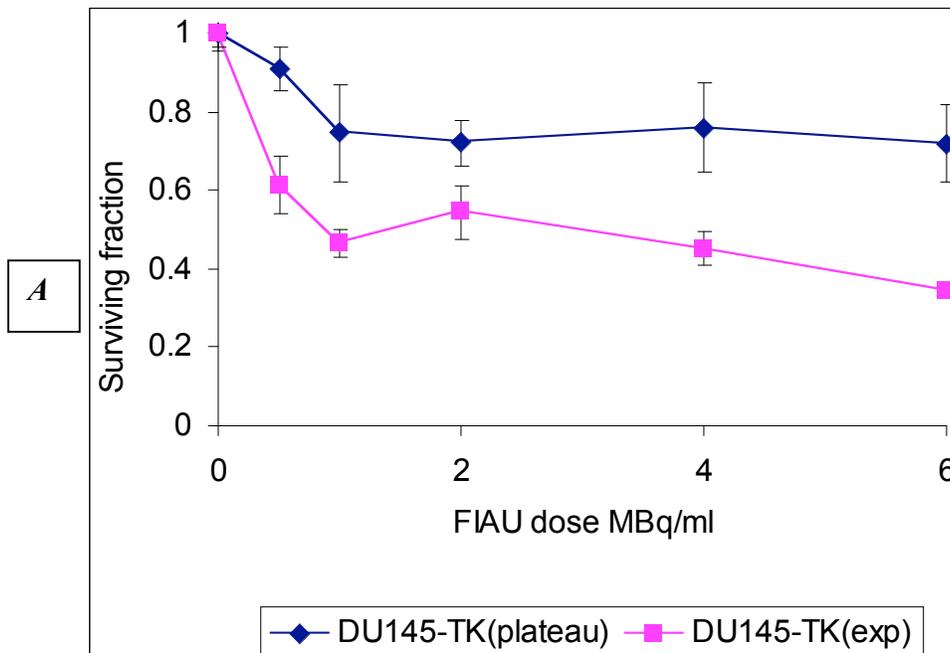
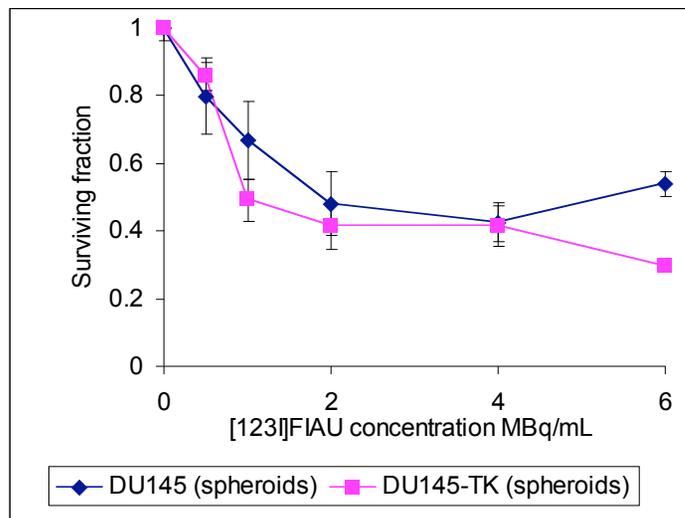


Figure 4.4: Clonogenic survival of the cell lines DU145-TK, *A* and 143B-TK, *B* treated with [¹²³I]FIAU for 4 h at either plateau or exponential phase. Data represent mean and s.d. of triplicate experiments.

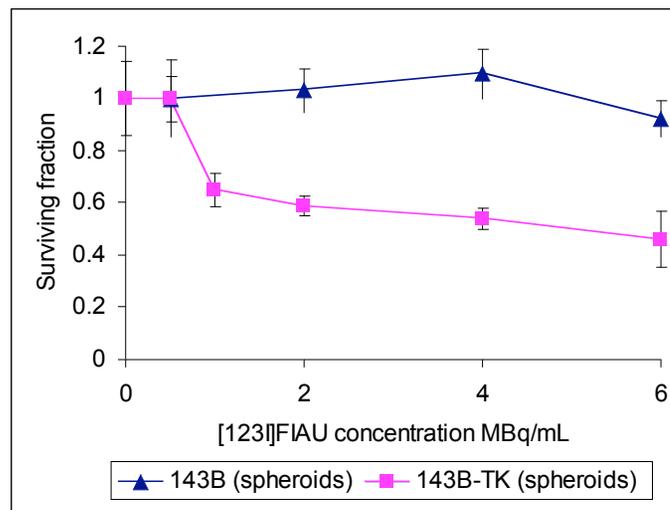
4.4.4 [¹²³I]FIAU sensitivity of multicellular spheroid models determined by clonogenic assay

When the spheroids of parental and TK-positive clones of DU145 cell line were treated with [¹²³I]FIAU for 4 h, sensitivity was shown to be similar to the monolayers and hence there was no enhanced sensitivity to [¹²³I]FIAU. The IC₅₀ values were similar to the values obtained with the treatment of monolayers of parental and TK positive DU145 (Fig. 4.5A). The IC₅₀ of the parental cell line monolayers and spheroids were 4.65 (±0.7) MBq/ml and 3.2 (±0.4) MBq/ml, respectively ($P > 0.05$) and for the TK-positive clone the IC₅₀ were 0.89 (±0.05) MBq/ml for monolayers and 0.91 (±0.06) for the spheroids ($P > 0.05$). Similar results were obtained with the control cell line 143B-TK for which the IC₅₀ increased from 1.86 (±0.59) to 4.3 (±0.61) MBq/ml with no statistical significance ($P = 0.069$) (Fig. 4.5B). However, the spheroids of the cell line UVW-TK showed reduced sensitivity to [¹²³I]FIAU compared with the monolayers where the IC₅₀ increased from 2.46 (± 0.13) MBq/ml to 6.4 (± 0.23) MBq/ml ($P = 0.008$) (Fig. 4.5C).

A



B



C

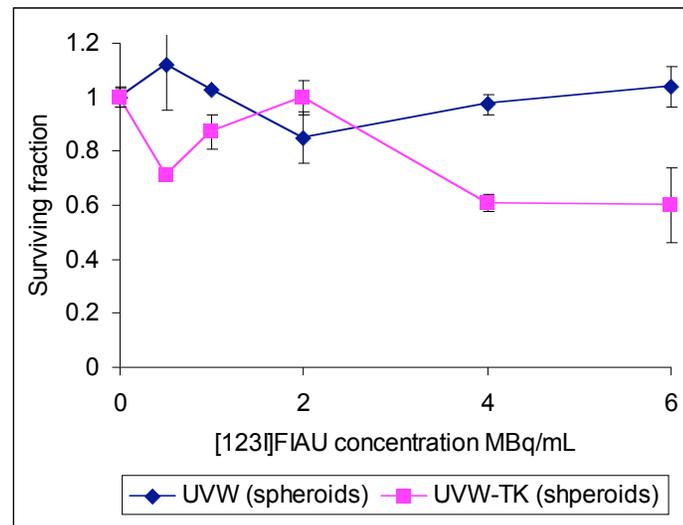


Figure 4.5: Clonogenic survival of spheroids of parental and TK-positive DU145 (A), 143B (B) and UVW (C) treated with increasing doses of [¹²³I]FIAU for 4 hours. Data represent mean and s.d. of triplicate experiments.

4.4.5 [¹²³I]FIAU sensitivity after treatment period equal to full doubling time of the treated cell lines.

When the [¹²³I]FIAU exposure period of the parental and TK-positive clones of all cell lines was increased to a period equal to their doubling times, a two log cell kill was observed (Fig. 4.6). The IC₅₀ values were reduced significantly compared to those obtained by the 4 h exposure. For example, the IC₅₀ for the clone 145-TK was reduced from 0.89 (±0.05) MBq/ ml to 0.018 MBq/ ml when the incubation period was increased from 4 h to 26 h (*P* < 0.001). There was also a rise in the TK sensitivity enhancement factor (EF) for the cell line DU145-TK from 5.2 after 4 h exposure to 7.67 after 26 h exposure (Tables 4.1 & 4.3). The treatment of DU145-TK and 143B-TK with equivalent molar concentration of unlabelled FIAU for the same period did not result in any growth inhibition (Fig. 4.6). In view of the short half life of ¹²³I (13 h), longer incubation periods will result in a smaller number of disintegrations towards the end of the incubation period. Because this study did not compare different isotopes of iodine, the number of disintegrations was not corrected for the incubation periods.

Table 4.3: IC₅₀ (kBq/ml) of [¹²³I]FIAU of parental and TK-positive DU145 after an incubation period equivalent to the cellular doubling time.

Cell line	IC ₅₀ (s.d)	EF	<i>P</i> value
DU145	142.08(26.3)		
DU145-TK	18.51(0.41)	7.67	0.0012
143B	ND		
143B-TK	42 (3.2)	ND	<0.001

ND, not determined.

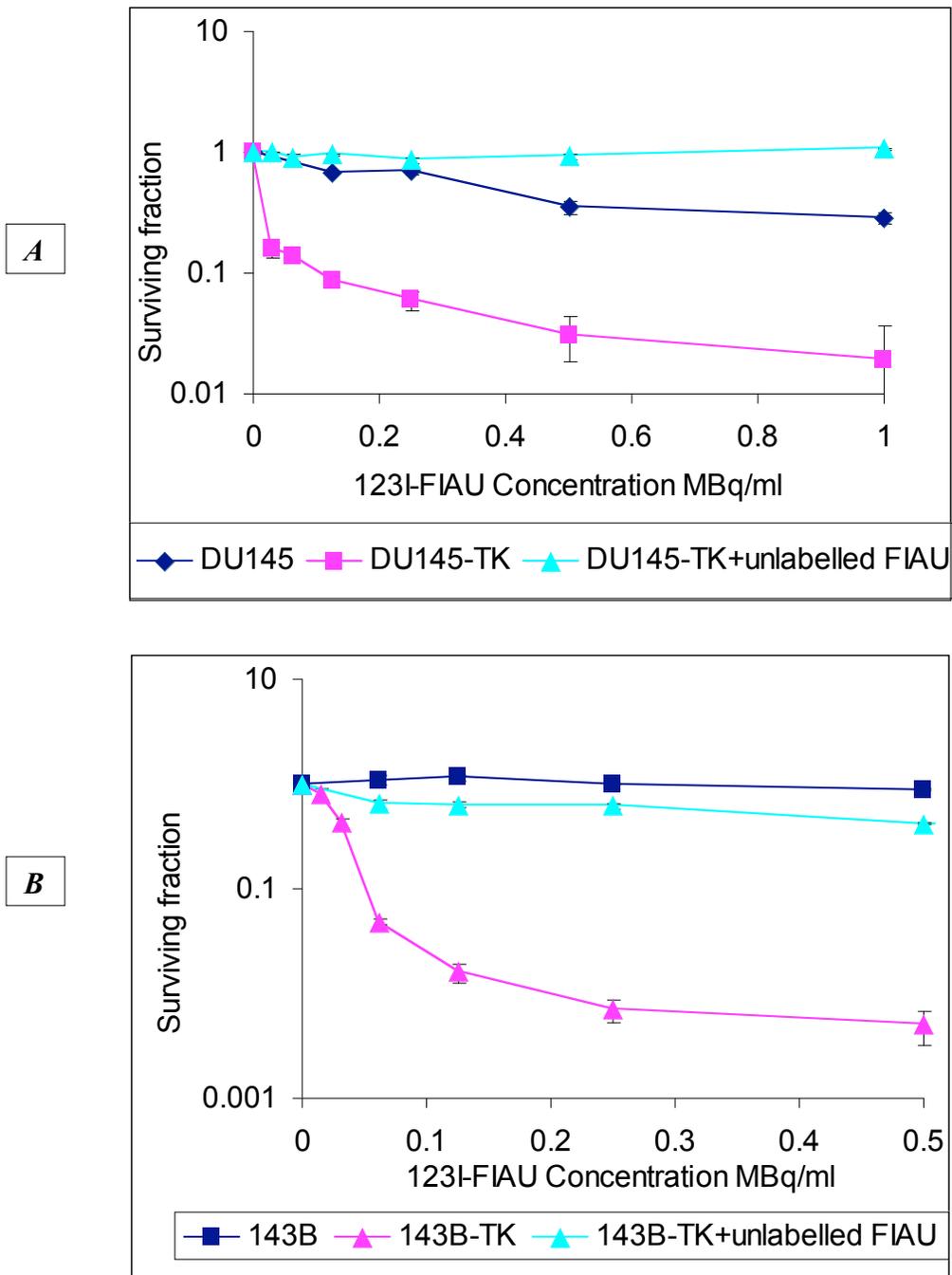


Figure 4.6: Clonogenic survival of the parental and TK-positive clones of the cell lines DU145 (A) and 143B (B) treated with various doses of [¹²³I]FIAU for a period equal to their doubling time. Data represents mean and s.d. of triplicate experiments. Data also included the treatment of the transfected clones of these two cell lines with the unlabelled FIAU.

4.4.6 Cell cycle analysis of TK-positive cells treated with [¹²³I]FIAU

The figures 4.7 and 4.8 show the cell cycle analysis of the cell line DU145-TK treated with [¹²³I]FIAU for 4 h and 26 h, respectively. The incubation period of 4 h had no effect on the cell cycle progression over the whole range of [¹²³I]FIAU doses (Fig. 4.7 & 4.9A). For example, the maximum [¹²³I]FIAU dose of 3 MBq/ml, which resulted in a survival fraction (SF) of 0.16 caused insignificant reduction in the percentage of cells in G₁-phase from 63.5% (untreated cells) to 62.8% ($P = 0.9$). In contrast, the 26 h treatment period induced significant cell cycle arrest at G₂/M phase. This cell cycle redistribution was evident even with the smallest dose of 62.5 kBq/ml, which resulted in a SF of 0.28 (Fig. 4.8 & 4.9B). This dose caused a decrease in the proportion of cells in G₁-phase from 63.08% (untreated cells) to 38.95% ($P = 0.026$) and a rise in the proportion of cells in G₂/M-phase from 21.98% to 49.85% ($P = 0.002$).

Similar results were obtained from the osteosarcoma cell line 143B-TK as shown in figure 4.10. The smallest dose of 62.5 kBq/ml reduced the proportion of cells in G₁-phase from 59% to 43.3% and increased the proportion of cells at G₂/M phase from 26.8% to 44.1%.

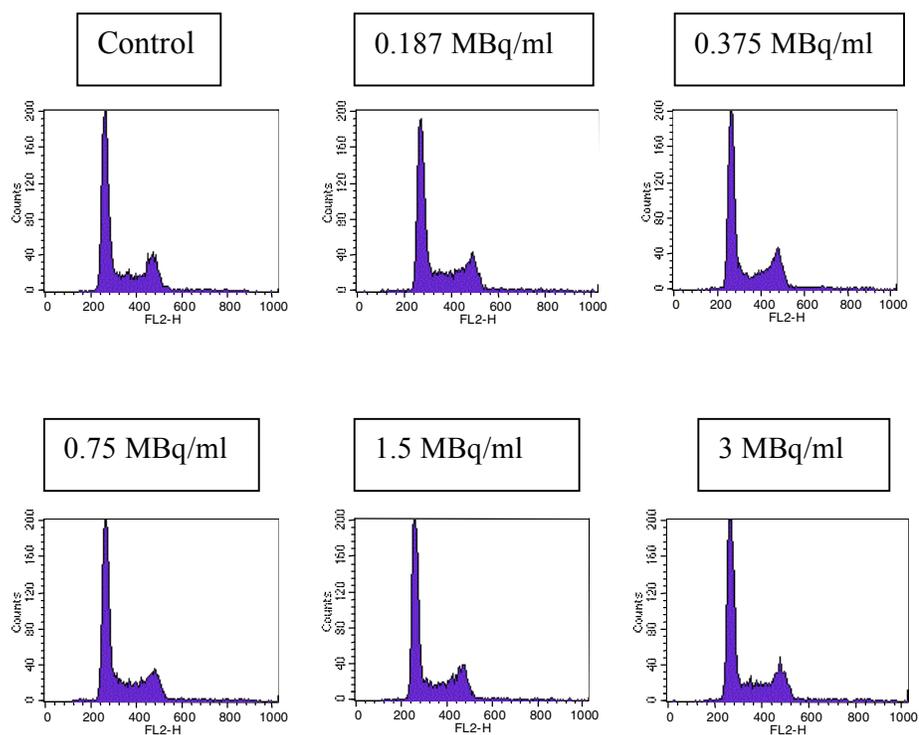


Figure 4.7: Cell cycle analysis for the cell line DU145-TK treated with increasing doses of [¹²³I]FIAU for 4 hours. There were no significant cell cycle changes even with the highest dose of 3 MBq/ ml.

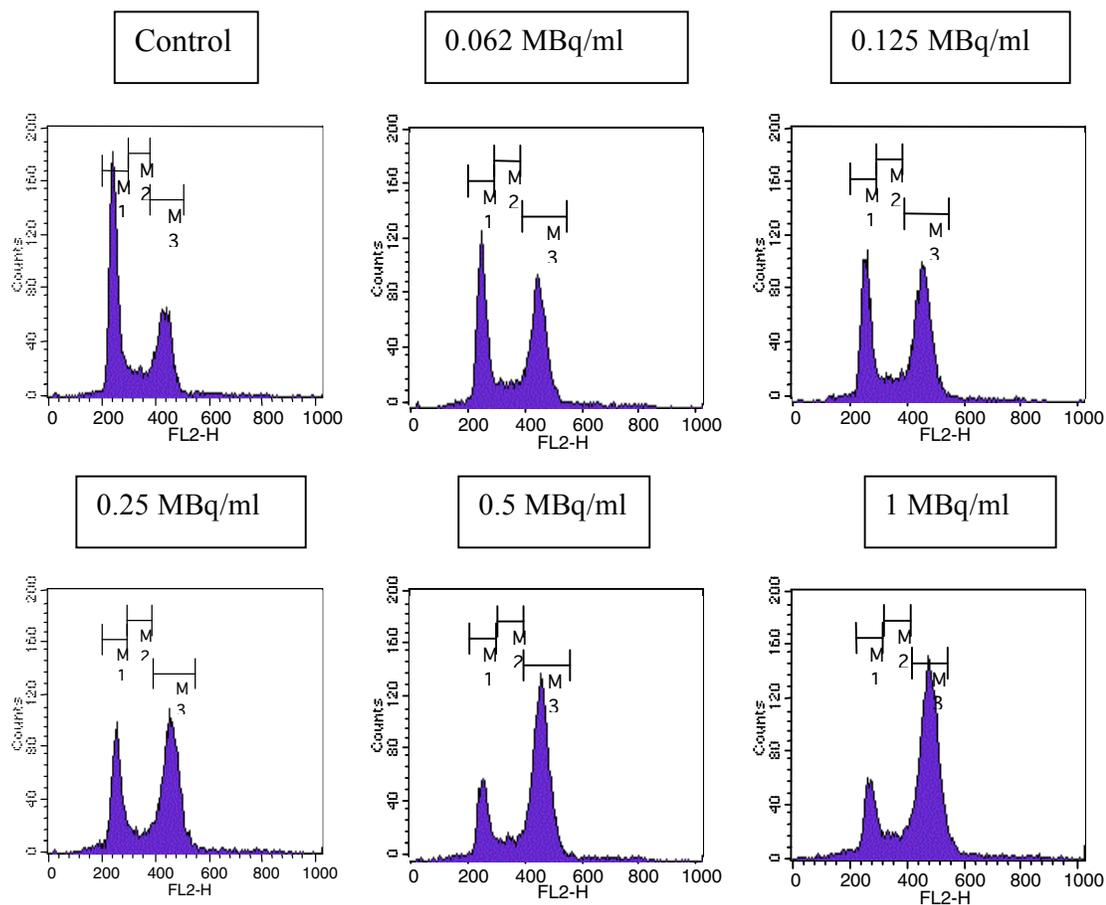


Figure 4.8: Cell cycle analysis for the cell line DU145-TK treated with increasing doses of [¹²³I]FIAU for 26 h showing evidence of cell cycle arrest at G2/M phase. M1, G0/G1; M2, S; M3, G2/M.

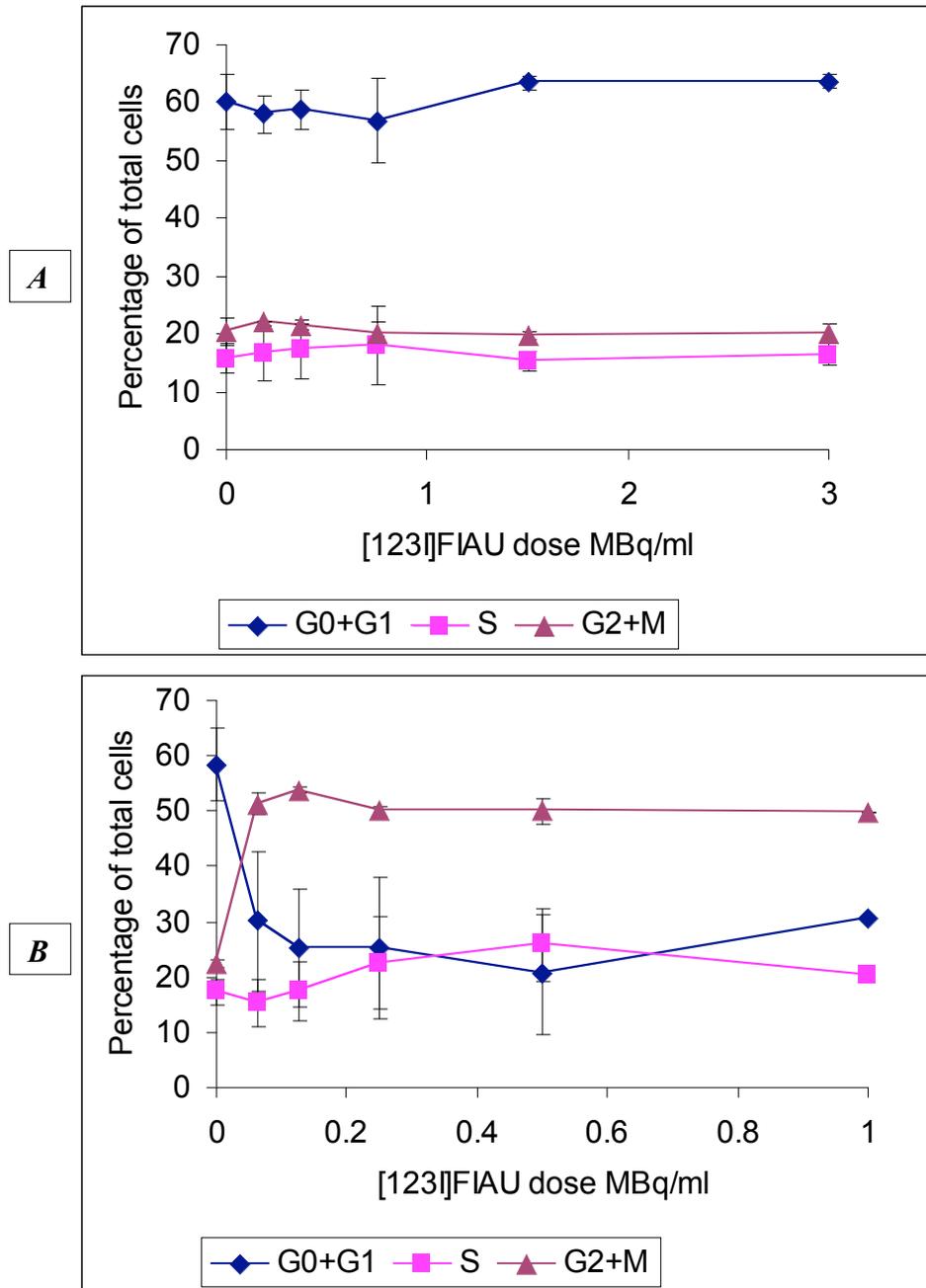


Figure 4.9: Cell cycle changes of the cell line DU145-TK treated with increasing doses of [¹²³I]FIAU for 4 h, *A* and 26 h, *B*. Data represent mean and s.d. of 2 independent experiments.

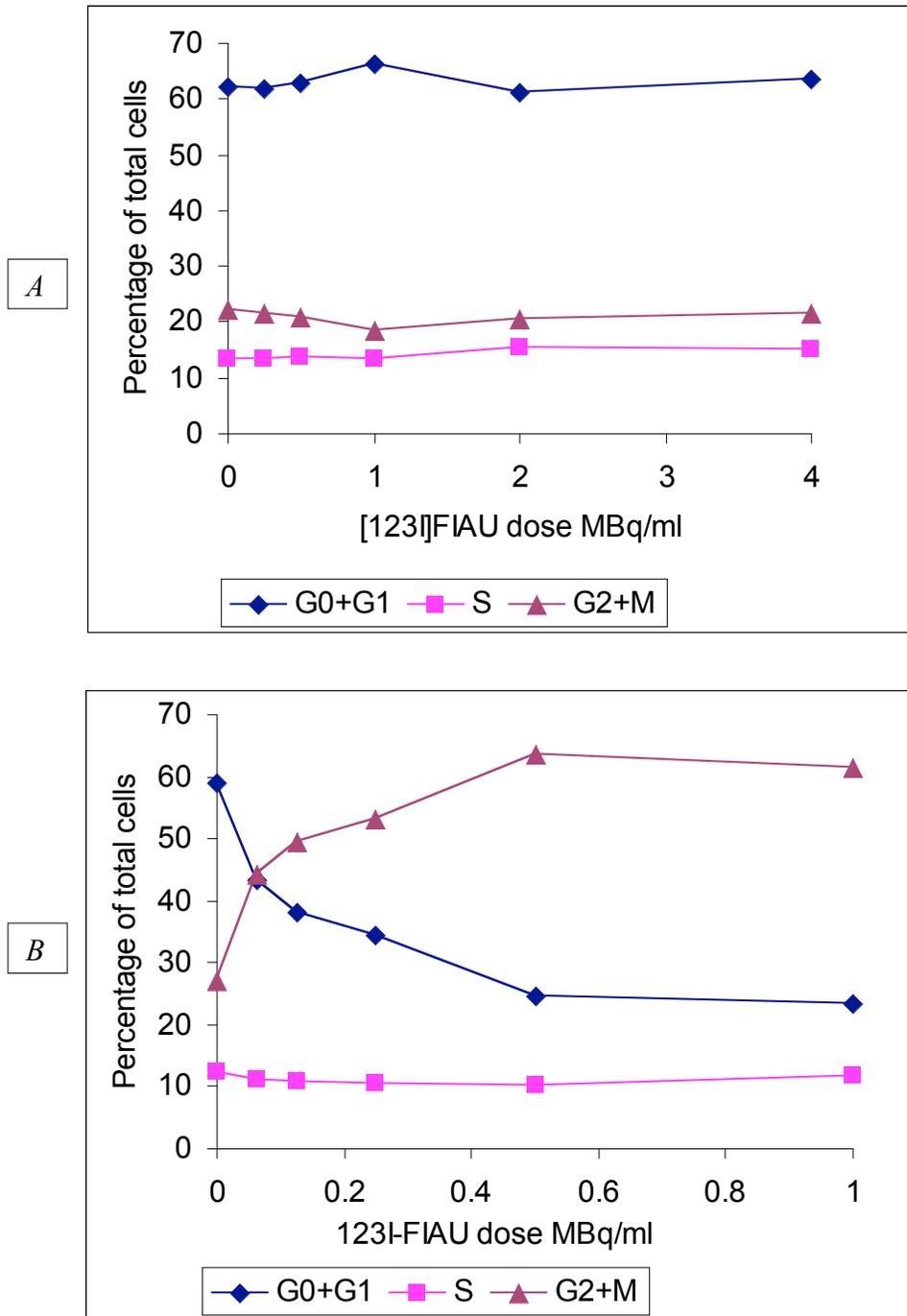


Figure 4.10: Cell cycle changes of the cell line 143B-TK treated with increasing doses of $[^{123}\text{I}]\text{FIAU}$ for 4 h, *A* and 20 h, *B*.

4.5 Discussion

The main purpose of the experiments described in this chapter was to explore the potential anticancer effect of radiolabelled FIAU on tumour cells bearing HSV1-*tk* gene. Following the incubation of the three cell lines DU145, UVW and 143B with [¹²³I]FIAU for 4 h, both parental and TK positive clones of all cell lines demonstrated a degree of sensitivity except the TK-negative cell line 143B. As shown in previous studies, this indicates that [¹²³I]FIAU sensitivity is TK mediated. The IC₅₀ values ranged from 0.89 ± 0.05 MBq/ml for the cell line DU145-TK to 7.03 ± 0.61 MBq/ml for the cell line UVW. The transfection of tumour cells with HSV1-*tk* gene significantly enhanced the sensitivity of all cell lines to [¹²³I]FIAU. The EF values ranged from 2.8 to 5.2 after 4 h exposure. This was in agreement with the results of our [¹²³I]FIAU uptake studies where, for example, the [¹²³I]FIAU/ *methyl*-[³H]TdR uptake ratio was 4.25 (±0.15) times higher in the TK positive than in the parental clone of the cell line DU145 (see section 3.4.2.2). However, the EF values for GCV sensitivity were considerably higher, as shown in section 3.4.3.1. This is probably due to the longer incubation period of 72 h which was planned according to similar experiments carried out by other groups (e.g. (Haubner et al., 2000; Makinen et al., 2000) .

In contrast, a previous study of DU145 cells transfected with the noradrenaline transporter (NAT) gene demonstrated upto 8-fold enhancement of the active uptake of the radiopharmaceutical [¹³¹I]MIBG compared to the nontransfected cells. This resulted in dose-dependent toxicity with statistically significant difference in the SF between the parental cells and the NAT transfected cells (Fullerton et al., 2004). In this study the sensitivity enhancement was not determined by comparing the IC₅₀ values.

As shown in Fig. 4.5, the spheroid experiments failed to demonstrate better tumour cell kill with crossfire or bystander effect using [^{123}I]FIAU. The possible reasons for this are the short radiation range of the radionuclide ^{123}I and the relatively short exposure period of the spheroids to FIAU. Neshasteh-Riz et al., compared the effect of ^{123}I -IUdR on spheroids of the glioma cell line UVW with that of ^{125}I -IUdR and ^{131}I -IUdR. The survival curves showed a dose dependent region at low concentration for all three pharmaceuticals, and a dose-independent region for high concentration of [^{125}I]IUdR and [^{123}I]IUdR. [^{123}I]IUdR was less effective than [^{125}I]IUdR over the whole concentration range (Neshasteh-Riz et al., 1998). In addition, another study suggested that tumour cells growing as spheroids manifest a reduction in division rate among cycling cells as well as a reduction in the proportion of cells in cycle (Neshasteh-Riz et al., 1997).

The prolongation of [^{123}I]FIAU exposure of the tumour cell lines to a period equal to their doubling times (Fig 4.6) caused a dramatic reduction in the clonogenic survival of these cells when the entire cell population goes through a full cell cycle. This, together with the reduced [^{123}I]FIAU sensitivity of cells in plateau phase, supports the evidence that this strategy works only on cycling tumour cells and in particular cells undergoing DNA synthesis. The clonogenic survival curves of the TK positive clones showed biphasic pattern of dose-dependent kill at low [^{123}I]FIAU concentrations and thereafter in the second phase no further increase in kill despite increasing radioactivity concentrations. This implies the presence of resistant subpopulation of clonogens.

Figure 4.8 shows that this prolonged exposure period has also resulted in cell cycle arrest at the G_2/M phases, in contrast to the negligible cell cycle changes observed after the 4 h exposure (Fig.4.7). This cell cycle arrest was evident even with the lowest dose

of 62 kBq/ ml. This event likely takes place at the repair check point after DNA radiation damage and before next cell division.

The arrest of cells at G₂-phase of cell cycle following DNA damage is observed readily in mammalian cells and has been studied by radiation biologists for decades, since before checkpoints were understood at the molecular level. The function of this checkpoint in normal cells is to prevent cells with damaged chromosomes from attempting the complex process of mitosis. The cells are arrested in G₂ to allow DNA repair to be completed. The controlling genes for this checkpoint have been identified in yeast, but not in mammalian cells (Hall, 2000). Further cellular division with damaged DNA will eventually result in cell death within 1 or 2 cell cycles, unless the cell manages to repair the damage before mitosis starts. Once the cells are arrested at this phase of the cell cycle, which is the most radiosensitive phase, they become vulnerable to a second insult of radiation or another TK substrate like GCV. This may have important implications for the scheduling of the GCV/FIAU combination therapy.

**Chapter 5 : Combination therapy with GCV and
[¹²³I]FIAU of tumour cells expressing HSV1-TK**

5.1 Aims

1. To evaluate the combination therapy effect of GCV and radiolabelled FIAU on tumour cells expressing the viral TK enzyme using the method of median effect analysis and combination index.
2. To determine the best timing schedule that leads to maximum synergism.
3. To highlight the possible underlying mechanisms of the end result of the different schedules of this combination therapy using cell cycle analysis.

5.2 Introduction

5.2.1 Rationale for the combination therapy

One of the main goals of any combination therapy in oncology is to reduce the required doses of each single treatment agent, thereby minimising the individual adverse effects. This is beneficial even if the outcome of the combination therapy is merely an additive effect. As a basic principle in oncology, additive or even synergistic antitumour action can be achieved by combining agents that have demonstrated single-agent activity and non-cross-resistance to overlap against drug-resistant subpopulation of tumour cells. In addition, these agents should have non-antagonistic and preferably additive or synergistic mechanisms of actions and non-overlapping toxicity profiles (Balis et al., 1997). As a proof of effectiveness, we have confirmed that radiolabelled FIAU is lethal to tumour cells with and without viral TK expression (chapter 4). GCV is taken up by all

cells but only phosphorylated and retained in cells that express TK gene (Frank et al., 1989). Both GCV and FIAU are selective substrates of the viral TK enzyme.. However, their dose-dependent adverse effects seem to be markedly different in terms of severity and affected organs.

The most serious side effect of the non-radiolabelled FIAU was the fatal hepatotoxicity which was reported after clinical trials involving treatment of chronic active viral hepatitis B (Brahams, 1994; Marshall, 1994). Systemic administration of GCV, however, was considerably safer when a large dose of 5 mg/ kg twice daily was administered for 14 days in the first 2 clinical trials of HSV1-TK/GCV gene therapy (Ram et al., 1997; Sterman et al., 1998). In both studies the tumour responses were low in number or of short duration.

However, higher doses of GCV may not be tolerated because of its toxic effects on the bone marrow, gut and kidneys (Wildner et al., 1999). Furthermore, simply increasing HSV1-TK enzyme level per cell above a minimal threshold level is not effective in augmenting the HSV1-TK/GCV system (Elshami et al., 1997).

Therefore, new strategies to circumvent these limitations, such as combination therapy, are urgently sought. Radiation therapy has traditionally played a major role in the combination therapy of human cancer together with surgery and/ or chemotherapy. More recently, encouraging results have been reported from an early phase clinical trial combining protocols of suicide gene therapy with external beam radiotherapy of prostate cancer (Freytag et al., 2003). We are here aiming to couple the Auger electron particle targeted-radiation cytotherapy by [¹²³I]FIAU with the suicide gene therapy HSV1-TK/GCV system in treating two different cell lines derived from prostate cancer and osteosarcoma.

5.2.2 Principle of median effect analysis and combination index

In 1984 Chou and Talalay introduced the median effect analysis in order to analyse and normalise all types of dose-response results by a uniform method (Chou, 1991; Chou & Talalay, 1984). This analysis is based on the median effect principle of the mass action law (Chou, 1974; Chou, 1976; Chou & Talalay, 1977) and has already been shown to be simple to apply and useful in the analysis of complex biological systems.

5.2.2.1 The Median Effect Equation

The median effect equation states that

$$f_a/f_u = (D/D_m)^m \quad (1)$$

Where D is the dose, f_a and f_u are, respectively, the fractions of the system affected and unaffected by the dose D , D_m is the dose required to produce the median effect (analogous to the more familiar IC_{50} , ED_{50} , or LD_{50} values, and m is a Hill-type coefficient signifying the sigmoidicity of the dose-effect curve, i.e., $m = 1$ in hyperbolic (first order or Michaelis-Menten) systems.

Since by definition, $f_a + f_u = 1$, an alternative to equation 1 is:

$$f_a/(1 - f_a) = (D/D_m)^m$$

and thus:

$$D = D_m [f_a/(1-f_a)]^{1/m}$$

5.2.2.2 The median effect plot

The median effect equation (equation 1) may be linearised by taking the logarithms of both sides, i.e.

$$\log [(f_a)^{-1} - 1]^{-1} = m \log (D) - m \log (D_m)$$

or
$$\log (f_a/f_u) = m \log (D) - m \log (D_m)$$

or $\log [(f_u)^{-1} - 1] = m \log (D) - m \log (D_m)$

Thus, in the median effect plot (e.g., Fig. 5.2)

$$y = \log (f_a/f_u)$$

$$x = \log (D)$$

The plot gives the slope, m , and the intercept of the dose effect plot with the x axis of $\log (D)$ which gives $\log (D_m)$ and consequently the D_m value [i.e. when $f_a = f_u$, $f_a/f_u = 1$ and hence $y = \log (f_a/f_u) = 0$]. Any cause consequence relationship that gives a straight line for this plot will provide the two basic parameters, m and D_m , and thus, the apparent equation that describe such a system. The linearity of the median-effect plot (as determined from linear regression coefficients) determines the applicability of the present method (Chou & Talalay, 1984).

5.2.2.3 Equations for the effect of multiple drugs

A systematic analysis in enzyme kinetic systems using the basic principles of mass-action law has led to the derivation of generalised equations for multiple inhibitors or drugs (Chou & Talalay, 1977; Chou & Talalay, 1981).

For two drugs (D_1 and D_2) that obey higher order conditions (Hill-type) in which each drug has a sigmoidal dose-effect curve (i.e., has more than one binding site or exhibits positive or negative cooperativity), if the effect of each drug is mutually exclusive (i.e., they have different modes of action or act independently), the generalised equation is:

$$\begin{aligned} [(f_a)_{1,2} / (f_u)_{1,2}]^{1/m} &= [(f_a)_1 / (f_u)_1]^{1/m} + [(f_a)_2 / (f_u)_2]^{1/m} \\ &= (D)_1 / (ED_{50})_1 + (D)_2 / (ED_{50})_2 \end{aligned} \quad (2)$$

Where, ED_{50} is the concentration of the drug that is required to produce a 50% effect.

If the effects of two drugs are mutually nonexclusive, then this relationship becomes:

$$[(f_a)_{1,2} / (f_u)_{1,2}]^{1/m} = [(f_a)_1 / (f_u)_1]^{1/m} + [(f_a)_2 / (f_u)_2]^{1/m} + [(f_a)_1 (f_a)_2 / (f_u)_1 (f_u)_2]^{1/m}$$

$$= (D)_1 / (ED_{50})_1 + (D)_2 / (ED_{50})_2 + (D)_1 (D)_2 / (ED_{50})_1 (ED_{50})_2 \quad (3)$$

In the special case where $(f_a)_{1,2} = (f_u)_{1,2} = 0.5$, equation 2 becomes:

$$(D)_1 / (ED_{50})_1 + (D)_2 / (ED_{50})_2 = 1 \quad (4)$$

This describes the ED50 isobologram.

Similarly equation 3 becomes:

$$(D)_1 / (ED_{50})_1 + (D)_2 / (ED_{50})_2 + (D)_1 (D)_2 / (ED_{50})_1 (ED_{50})_2 = 1 \quad (5)$$

Which does not describe an isobologram, because of the additional term on the left.

5.2.2.4 Quantitation of synergism, summation and antagonism

When experimental results are entered into equations 2 & 3, if the sum is greater than, equal to, or smaller than 1, it may be inferred that antagonism, summation, or synergism of effects, respectively, has been observed. Therefore, from equations 2 & 3, if the observed combined effect is greater than the calculated additive effect, $(f_a)_{1,2}$, synergism is indicated; if it is smaller, antagonism is indicated.

It is, however, convenient to designate a “combination index” (CI) for quantifying synergism, summation and antagonism, as follows:

$$CI = (D)_1 / (D_x)_1 + (D)_2 / (D_x)_2 \quad (6)$$

for mutually exclusive drugs, and

$$CI = (D)_1 / (D_x)_1 + (D)_2 / (D_x)_2 + (D)_1 (D)_2 / (D_x)_1 (D_x)_2 \quad (7)$$

for mutually nonexclusive drugs.

For mutually exclusive or nonexclusive drugs,

when $CI < 1$, synergism is indicated.

$CI = 1$, summation is indicated.

CI > 1, antagonism is indicated.

To determine synergism, summation and antagonism at any effect level (i.e. for any f_a value), the procedure involves three steps: i) construct the median effect plot which determines m and D_m values for drug 1, drug 2 and their combination; ii) for a given degree of effect (i.e. a given f_a value represents x percent affected), calculate the corresponding doses [i.e. $(D_X)_1$, $(D_X)_2$ and $(D_X)_{1,2}$] by using the alternative form of equation 1, $D_X = D_m [f_a / (1 - f_a)]^{1/m}$; iii) calculate the combination index (CI) by using equations 6 or 7, where $(D_X)_1$ and $(D_X)_2$ are from step (ii), and $(D_X)_{1,2}$ [also from step (ii)] can be dissected into $(D)_1$ $(D)_2$ by their known ratio, P/Q. Thus, $(D)_1 = (D_X)_{1,2} \times P/(P+Q)$ and $(D)_2 = (D_X)_{1,2} \times Q/(P+Q)$ (Chou & Talalay, 1984).

5.3 Materials and Methods

5.3.1 Cell lines

Two HSV1-TK-positive cell lines, the prostate cancer cell line DU145-TK and the osteosarcoma cell line 143B-TK cell line, were used in this study because of the high stability of HSV1-TK gene expression.

5.3.2 Timing schedules of the combination therapy

For all treatment schedules, cells were treated in 25 cm flasks. Cells in exponential phase were seeded at 10^5 cells/ flask, 48 h before the first dose. Individual agent therapies were also carried out simultaneously with each schedule and surviving fractions were calculated as a percentage of the untreated control of each schedule separately. It has been shown that the plasma half life of FIAU in human is about 4 h

(Feinberg et al., 1985; Haubner et al., 2000; King, 1991) and therefore the treatment period with [¹²³I] FIAU of tumour cells was determined according to this to mimic *in vivo* kinetics. The treatment period of GCV of 72 h was selected according to the results of previous studies which showed maximum cell kill after 72 h incubation (Haubner et al., 2000; Makinen et al., 2000) .

5.3.1.1 Treatment with GCV before FIAU

Cells in exponential phase were incubated in 4 ml medium containing various concentrations of GCV at 37°C. After 72 h, medium was removed, monolayers were washed with PBS and 1 ml of medium containing the appropriate doses of [¹²³I] FIAU was added to each flask and cells were further incubated for 4 h. Following this, cells were washed twice with PBS.

5.3.1.2 Treatment with GCV after FIAU

1 ml of medium containing various concentrations of [¹²³I] FIAU was added to each flask and incubated. After 4 h, medium containing [¹²³I] FIAU was removed; cells were washed twice with PBS and treated with the appropriate doses of GCV for 72 h and then washed twice with PBS.

5.3.1.3 Simultaneous Treatment with GCV and FIAU

Cells were initially incubated in 1 ml of medium containing both GCV and [¹²³I] FIAU in concentrations appropriate to each combination dose for 4 h. Medium was then removed and cells washed twice with PBS and 4 ml of medium containing the same concentration of GCV was added. Cells were then incubated for another 68 h and then washed twice with PBS.

At the end of each schedule, single drug or combination effects were determined by clonogenic assay as described in section 2.2.4.

5.3.3 Cell cycle analysis

The effect of GCV and [¹²³I]FIAU exposure on cell cycle phase distribution was assessed by the method described in section 2.2.6.

5.3.4 Statistical analysis

Median effect plots and combination indexes were generated by entering the data of clonogenic assays into a Microsoft Excel template using the method and equations described in section 5.2.2.4.

5.4 Results

5.4.1 Combination therapy of the cell line 143B-TK using various schedules of delivery of GCV and [¹²³I]FIAU

5.4.1.1 Calculating the combination doses of GCV and [¹²³I]FIAU

Clonogenic assays were carried out to determine the IC₅₀ values of individual therapies using GCV or [¹²³I]FIAU. The ratio of the concentrations of the two drugs used in combination therapy was determined according to the method described in Table 5.1. A constant ratio of 1 MBq/ml [¹²³I]FIAU to 25 ng/ml GCV was used for the whole range of doses. The combination ratio was designed to approximate the IC₅₀ of the two agents, so that the contribution of the effect for each drug in the mixture would be about the same (i.e., equipotency ratio), although any other ratios could also be used (Chou et al., 1994).

Table 5.1: Calculating individual and combination doses of GCV and [¹²³I] FIAU for the treatment of 143B-TK cell line. The combination doses are the sum of GCV and [¹²³I] FIAU doses and therefore they do not have a unit.

	[¹²³ I]FIAU MBq/ml	CGV ng/ml	
IC ₅₀	1.8	44.89	
Approximate ratio	1	25	
			Combination doses
dose 1	4	100	104
dose 2	2	50	52
dose 3	1	25	26
dose 4	0.5	12.5	13
dose 5	0.25	6.25	6.5

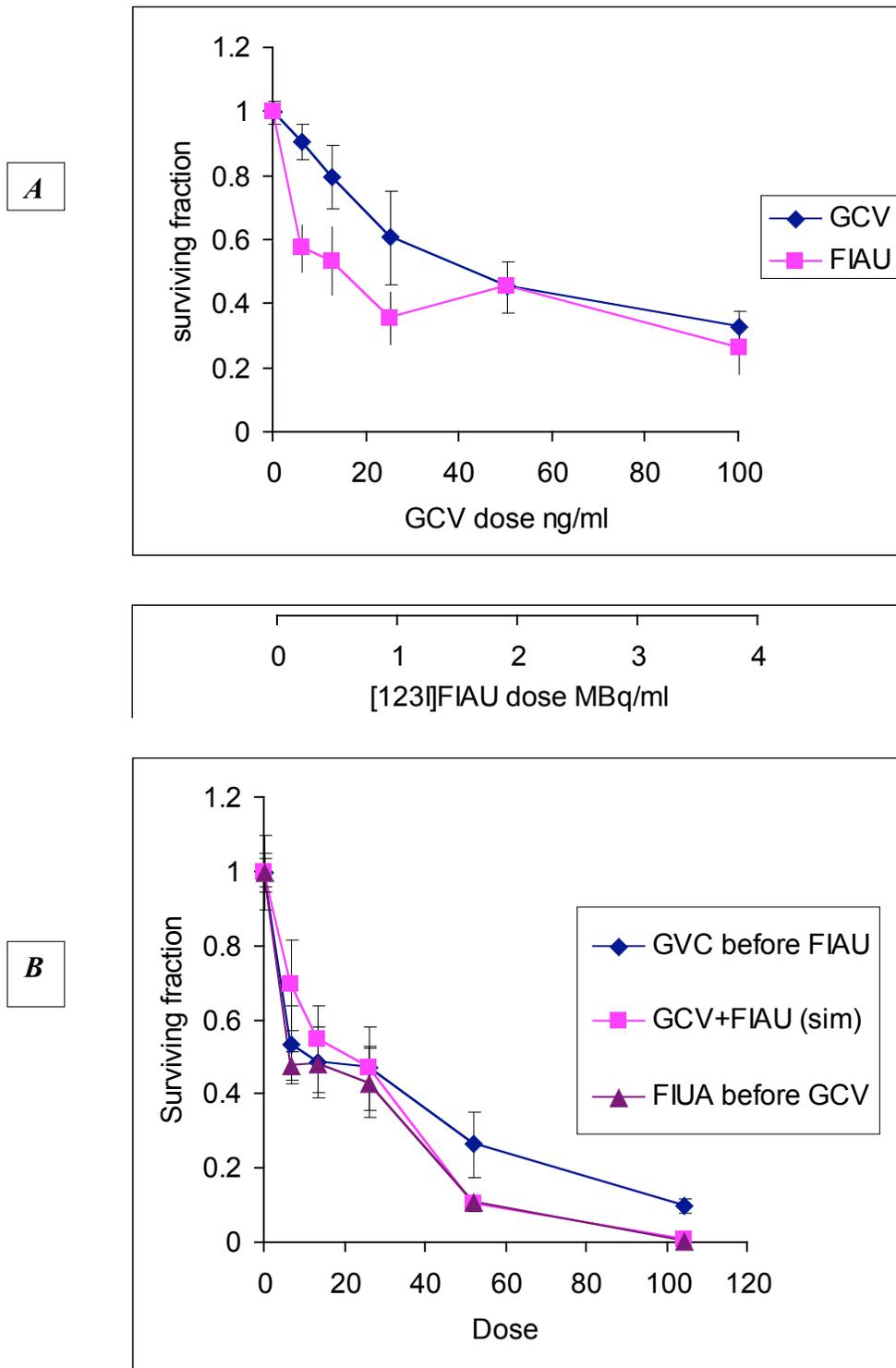


Figure 5.1: Clonogenic survival of single agent (A) and combination (B) therapies of 143B-TK cell line. Doses correspond to the concentrations determined in Table 5.1. Data represent the means and s.d of 6 observations of 2 experiments. Sim, simultaneous treatment.

5.4.1.2 The median effect plots of individual and combination therapies

Data obtained from the clonogenic assays of each single agent (Fig. 5.1A) and the different schedules of combination therapy (Fig. 5.1B) were used to construct the median effect plots shown in figures 5.2 and 5.3 using the logarithm of median effect equation as described in section 5.2.2. The dose-effect relationship parameters are shown in Table 5.2 from which the combination index values were determined as described in section 5.4.1.3 .

Table 5.2: Dose-effect relationship parameters of each treatment schedule for 143B-TK cells treated with GCV or/and FIAU as obtained from the median effect plots shown in figures 5.2 and 5.3.

Combination schedule	D_m		m	r
	GCV	FIAU		
	ng/ml	MBq/ml		
GCV	44.89		0.969	0.99
FIAU		0.62	0.432	0.865
GCV before FIAU	12.8	0.51	0.81	0.914
GCV+FIAU (sim)	14.61	0.58	1.33	0.936
FIAU before GCV	12.65	0.508	1.71	0.879

D_m , IC_{50} of the individual drug (antilog of x-intercept)

m , the slope

r , linear correlation coefficient of the dose-effect curve

Sim, simultaneous treatment

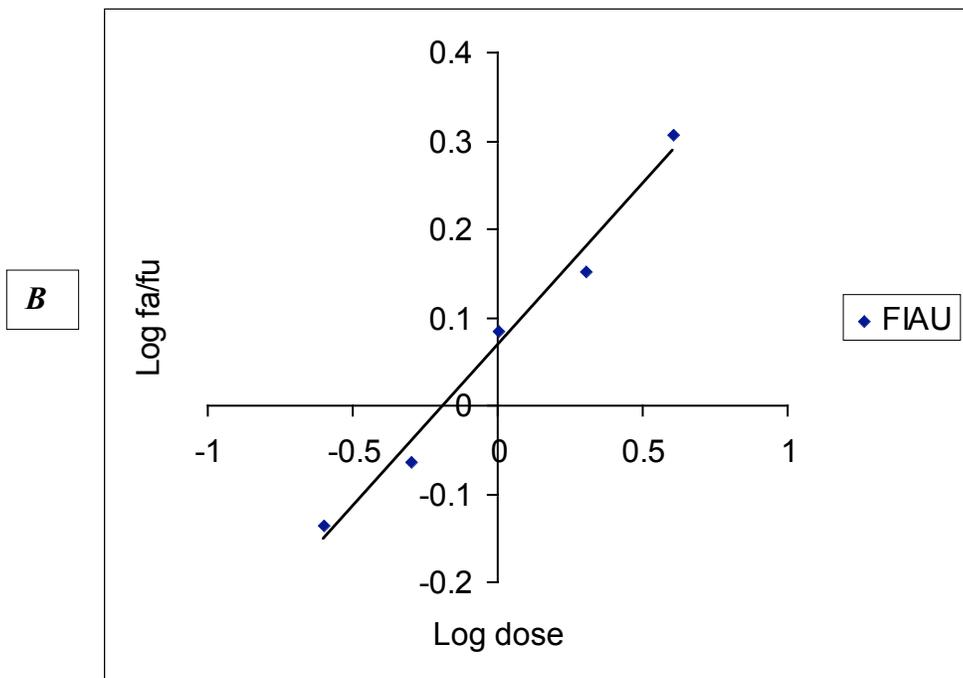
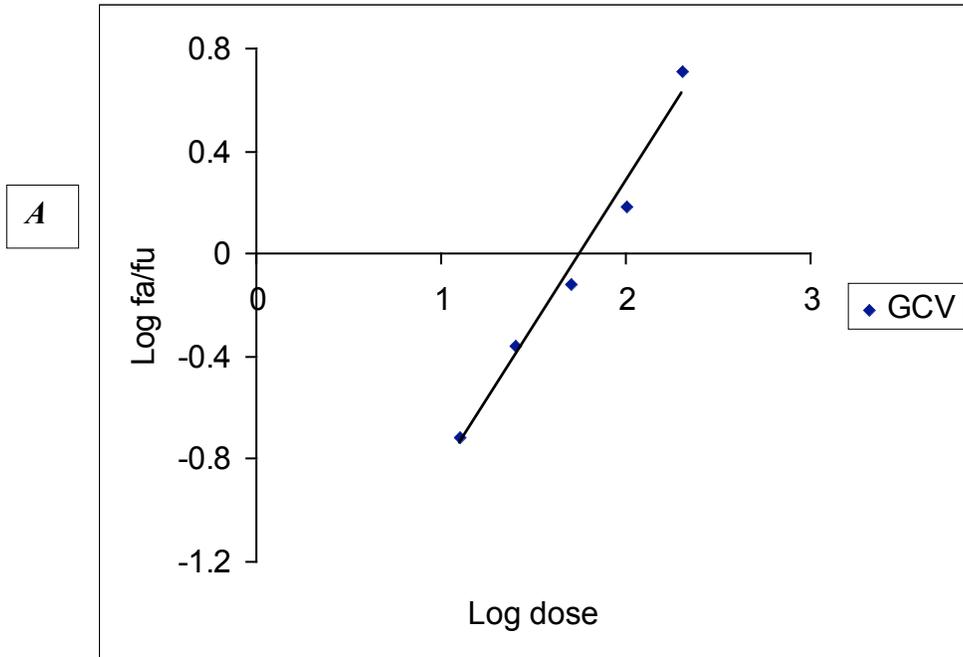


Figure 5.2: The median effect plots of individual agent treatment of the cell line 143B-TK.

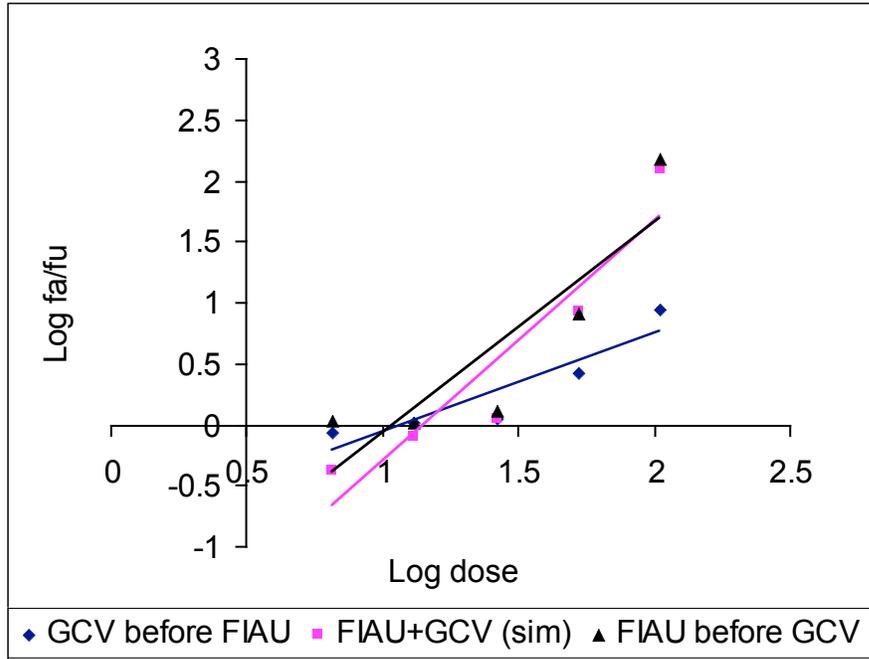


Figure 5.3: The median effect plots of the different schedules of combination therapy of 143B-TK cell line. Sim, simultaneous treatment.

5.4.1.3 Combination index values of different treatment schedules of 143B-TK cell line

All combination therapy schedules of this cell line showed evidence of synergism between GCV and [¹²³I]FIAU at the high toxicity concentration range regardless of the exclusivity of their effects. Doses which inhibited the growth of more than 50% of the cells caused a synergistic effect substantiated by CI values less than 1. As shown in Table 5.3, CI values at the 50% growth inhibition dose were close to 1, except the simultaneous GCV+[¹²³I]FIAU schedule, indicating additivity or summation when the effect was assumed to be mutually exclusive.

Table 5.3: Combination index values obtained from the different schedules of combination treatment of 143B-TK cell line with [¹²³I]FIAU and GCV. Values in parenthesis are CI generated assuming that the effects of combinations of drugs are mutually nonexclusive.

Combination schedule	Combination index (CI) values			
	IC ₃₀	IC ₅₀	IC ₇₀	IC ₉₀
GCV before FIAU	2.272 (2.7)	1.067 (1.26)	0.617 (0.71)	0.454 (0.48)
GCV+FIAU (sim)	3.9 (5.22)	1.26 (1.56)	0.531 (0.6)	0.256 (0.26)
FIAU before GCV	3.779 (5.18)	0.924 (1.31)	0.334 (0.43)	0.145 (0.157)

IC_x, concentration that inhibits the growth of x percentage of cells

Sim, simultaneously

5.4.2 Combination therapy of the cell line DU145-TK using various schedules of delivery of GCV and [¹²³I]FIAU

5.4.2.1 Calculating the combination doses of GCV and [¹²³I]FIAU

Table 5.4 shows the individual and combination doses for the cell line DU145-TK. The maximum combination dose was calculated according to a fixed ratio of 3 MBq/ml to [¹²³I]FIAU 1ug/ml GCV followed with serial two-fold dilutions for the smaller doses.

Table 5.4: Calculating individual and combination doses of GCV and [¹²³I] FIAU for the treatment of DU145-TK cell line

	FIAU	GCV	
	MBq/ml	g/ml	
IC₅₀	0.88	0.268	
Approximate ratio	3	1	
			Combination doses
Dose 1	0.375	0.125	0.5
Dose 2	0.75	0.25	1
Dose 3	1.5	0.5	2
Dose 4	3	1	4

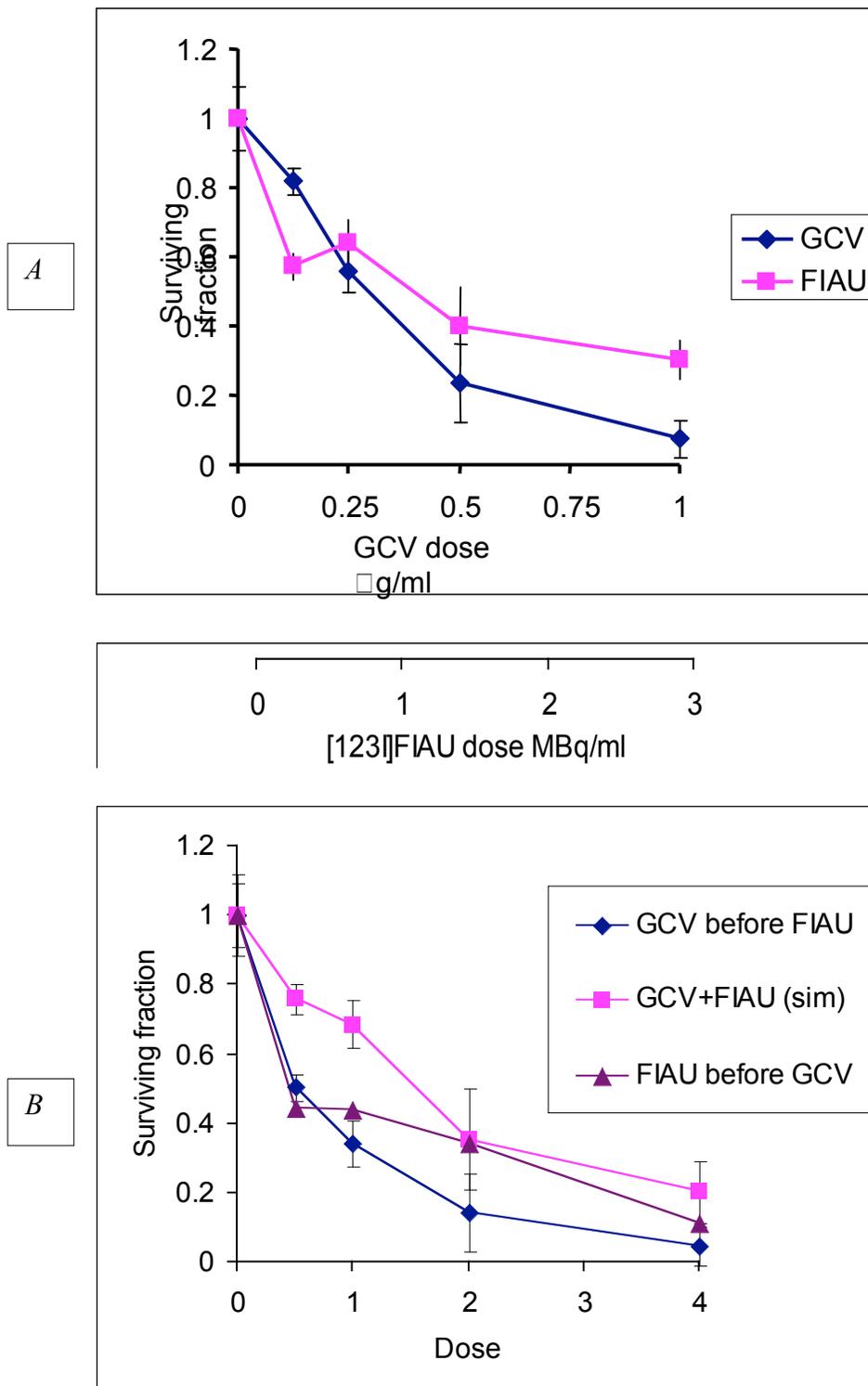


Figure 5.4: Clonogenic survival of single agents (A) and combination (B) therapies of DU145-TK cell line. Doses correspond to the concentrations illustrated in Table 5.4. Data represent the means and s.d of 6 observations of 2 experiments. Sim, simultaneous treatment.

5.4.2.2 The median effect plots of individual and combination therapies

The median effect plots for the cell line DU145-TK constructed as described in section 5.2.2 are shown in Fig. 5.5 & 5.6.

Table 5.5: Dose-effect relationship parameters of each treatment schedule for DU145-TK cell line treated with GCV or/and FIAU as obtained from the median effect plots shown in figures 5.5 and 5.6.

Combination schedule	D_m		m	r
	GCV	FIAU		
	g/ml	MBq/ml		
GCV	0.268		1.96	0.995
FIAU		0.88	0.599	0.854
GCV before FIAU	0.142	0.42	1.26	0.992
GCV+FIAU (sim)	0.229	0.68	0.871	0.831
FIAU before GCV	0.172	0.51	0.861	0.881

D_m , IC_{50} of the individual drug (antilog of x-intercept)

m , the slope

r , linear correlation coefficient of the dose-effect curve

Sim, simultaneously

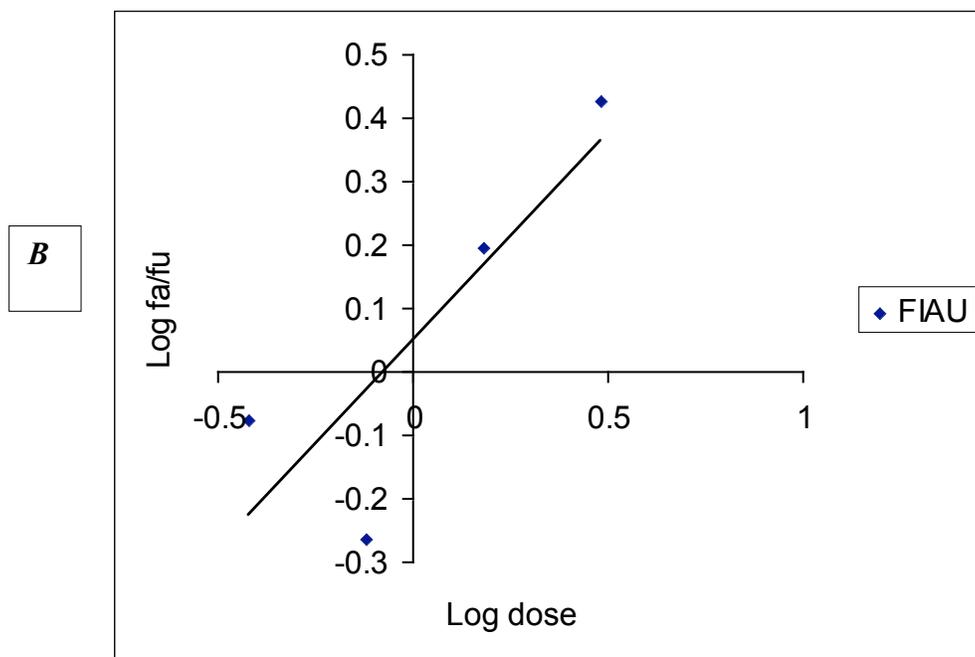
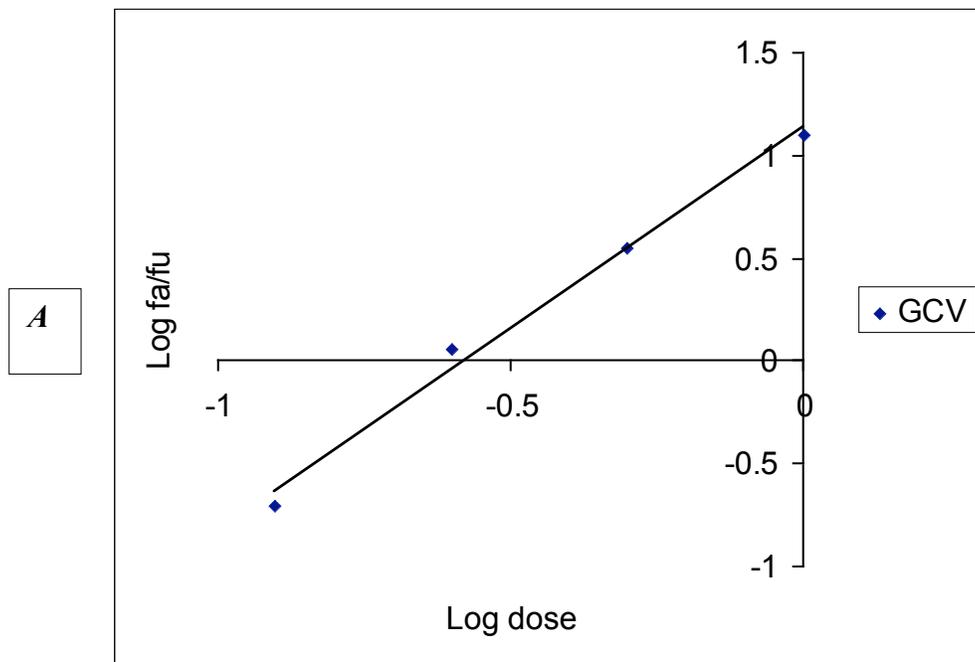


Figure 5.5: The median effect plots of individual agent treatment of DU145-TK cell line.

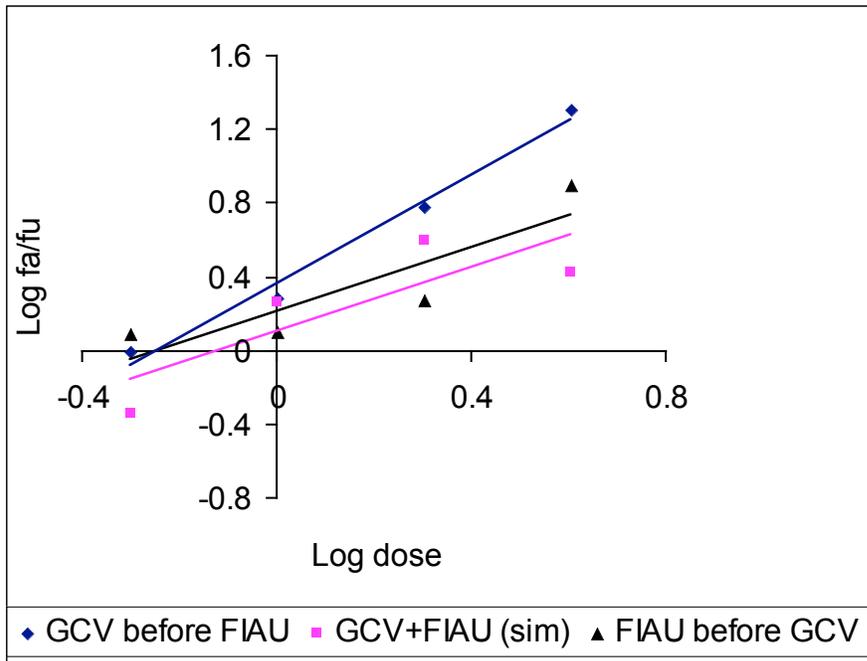


Figure 5.6: The median effect plots of the different schedules of combination therapy of DU145-TK cell line. Sim, simultaneous treatment.

5.4.2.3 Combination index values of various treatment schedules of DU145-TK cell line

Contrary to the findings of the other cell line 143B-TK, two of the combination schedules of this cell line showed evidence of antagonism between GCV and [¹²³I]FIAU with CI values greater than 1 throughout the whole range of doses (Table 5.6). One exception is the schedule of GCV before FIAU, where doses that caused 50% or greater growth inhibition revealed additive combination effect with CI values close to 1 when the combination drug effect was assumed as mutually exclusive.

Table 5.6: Combination index values obtained from the different schedules of combination treatment of DU145-TK cell line with [¹²³I]FIAU and GCV. Values in parenthesis are CI generated assuming that the effects of combinations of drugs are mutually nonexclusive.

Combination schedule	Combination index (CI) values			
	IC ₃₀	IC ₅₀	IC ₇₀	IC ₉₀
GCV before FIAU	1.439 (1.87)	1.025 (1.28)	0.92 (1.07)	1.083 (1.15)
GCV+FIAU (sim)	1.712 (2.32)	1.647 (2.32)	2.004 (2.75)	3.846 (4.73)
FIAU before GCV	1.274 (1.61)	1.24 (1.62)	1.521 (1.95)	2.947 (3.47)

IC_x, concentration that inhibits the growth of x percentage of cells

Sim, simultaneous treatment

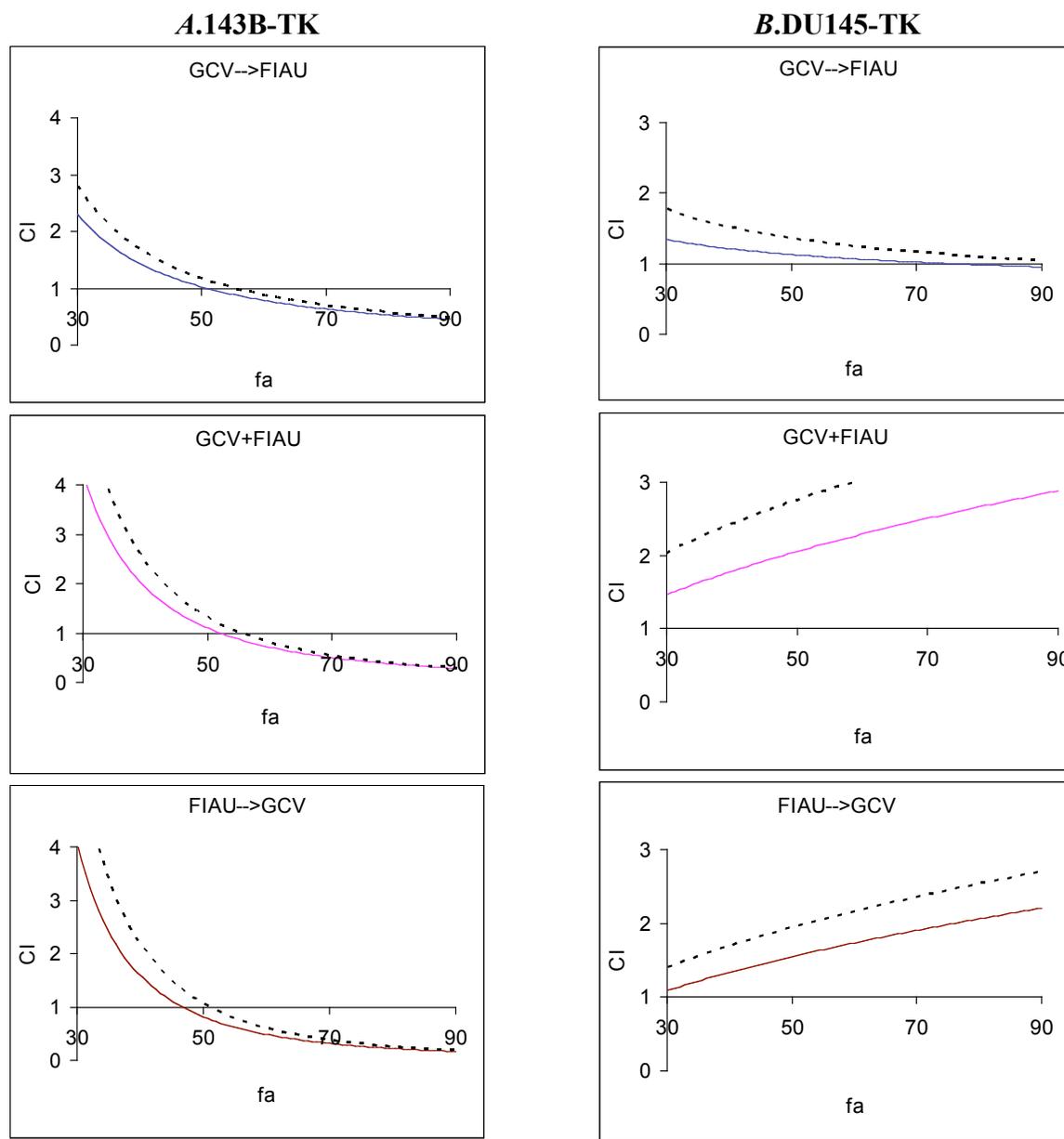


Figure 5.7: Comparison of combination index isobolograms of the cell lines 143B-TK (A) and DU145-TK (B). fa, affected fraction; CI, combination index. Solid lines and dotted lines indicate CI for mutually exclusive and nonexclusive mechanism of action, respectively, of the combination agents GCV and FIAU. Part of the curve above, overlapping or below X-axis (when CI=1) indicates antagonism, summation or synergism, respectively.

5.4.4 Effect of the treatment schedules on the cell cycle distribution

The cell cycle analysis of DU145-TK cell line following exposure to a range of GCV concentrations for 72h is shown in Figures 5.8 and 5.9A. The treatment with GCV alone resulted in a slight and dose-dependent increase in the proportion of cells in S- and G2/M-phases compared with the untreated cells. The GCV dose of 0.5 ug/ml, which corresponds to a SF of 0.21, induced a decrease in the proportion of cells in G1-phase from 64.4% to 53.3% ($P = 0.02$) and a rise in the percentage of cells in the S-phase from 13.6% to 18.1% ($P = 0.061$). Similarly, the proportion of cells in the G2/M phase increased from 18.4% to 23.8% ($P = 0.107$).

Similar cell cycle redistribution was observed in the 143B-TK cell line (Fig. 5.9B). For example, the GCV dose of 100 ng/ml, which caused a SF of 0.33, reduced the percentage of cells in G1-phase of the cell cycle from 65.3% to 56% and raised the percentage of cells in S-phase from 10.1% to 13.3% ($P = 0.08$).

Cell cycle changes after 4 h exposure to [¹²³I]FIAU are shown and discussed in chapter 4, section 4.4.6. As shown in Figures 4.9A and 4.10A, there was no significant change in the cell cycle distribution.

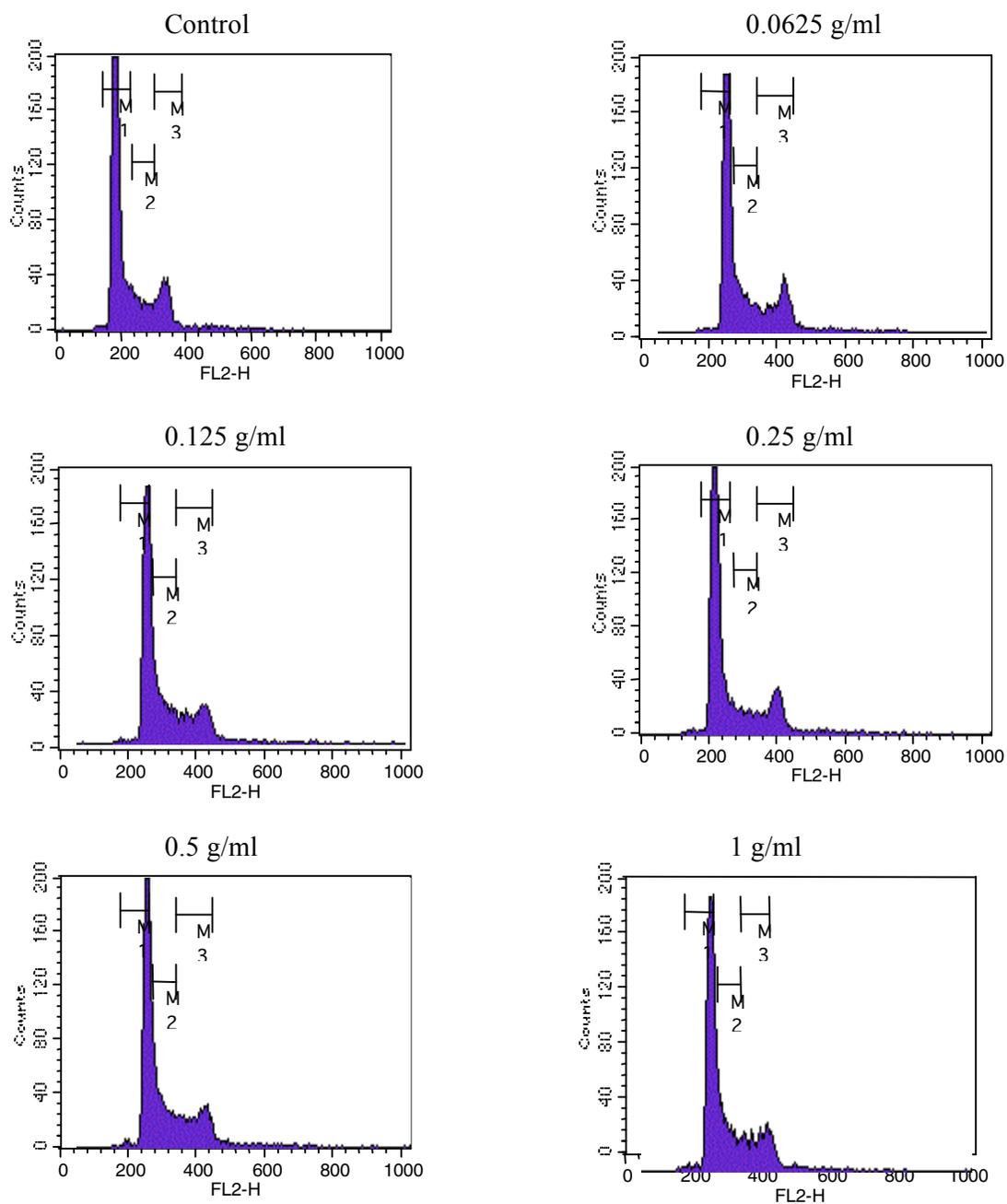
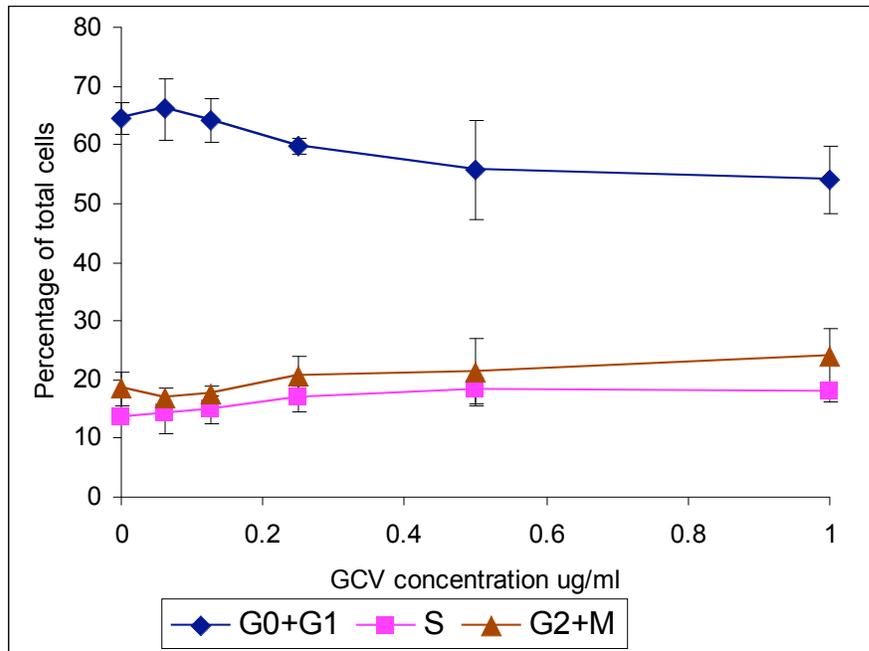


Figure 5.8: Cell cycle analysis for the cell line DU145-TK after treatment with increasing doses of GCV for 72 h. M1, M2 and M3 are the cell cycle phases of G1, S and G2/M, respectively.

A



B

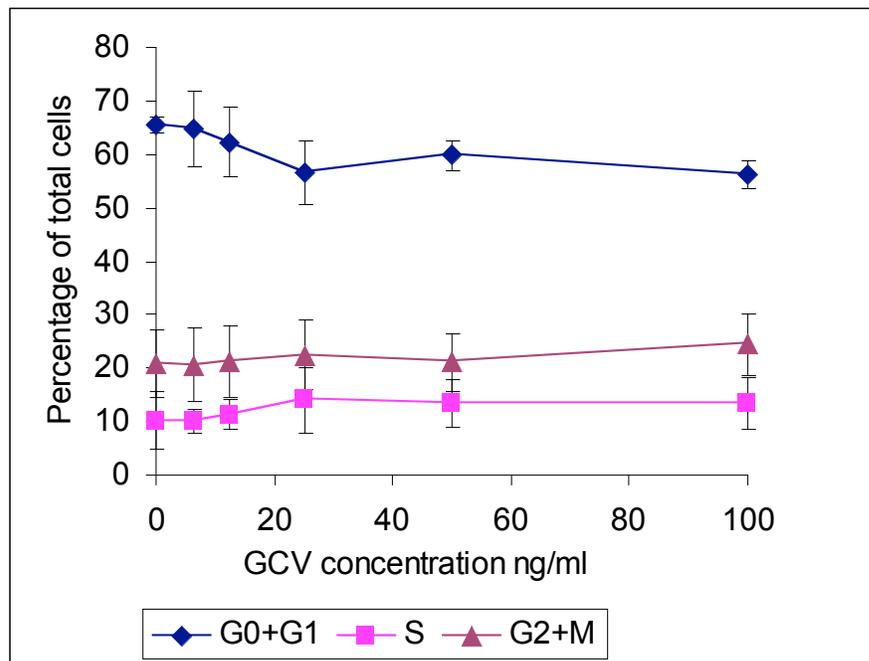


Figure 5.9: Cell cycle changes after treating the cell lines DU145-TK (A) and 143B-TK (B) with a range of doses of GCV for 72h. Data represent means and s.d. of 2 independent experiments.

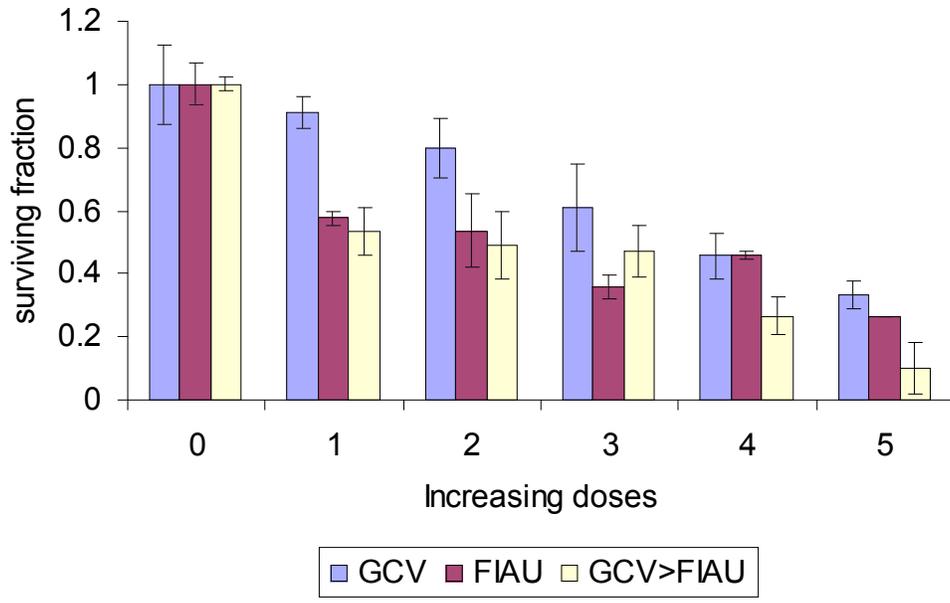


Figure 5.10: Comparison of the clonogenic survival of the individual and combination therapy of the cell line 143B-TK with GCV or/ and [¹²³I]FIAU for the combination schedule GCV before FIAU. Data represent means and s.d. of 6 observations from 2 experiments.

5.5 Discussion

This study was designed to assess the possibility of synergistic interaction between GCV and [^{123}I]FIAU in the treatment of tumour cells expressing the gene of HSV1-TK. One of the reasons for choosing the method of median effect analysis and combination index was that it requires a small number of experiments compared with the full isobologram analysis. This is particularly important if the combination involves a radiopharmaceutical drug in order to minimise the risk of radiation exposure and contamination.

We hypothesised that [^{123}I]FIAU toxicity to tumour cells expressing HSV1-TK would occur by two different pathways. The first results from HSV1-TK enzyme-mediated phosphorylation and subsequent DNA incorporation of [^{123}I]FIAU. This is similar to the mechanism of action of GCV and would lead to mutually exclusive drug effects in the GCV/ [^{123}I]FIAU combination. The other mechanism of [^{123}I]FIAU toxicity is the result of the ionising radiation due to the Auger electrons emitted by ^{123}I , leading to mutually non-exclusive interaction when these agents are combined. Therefore, CI values were generated according to both assumptions of similar and different mode of action.

The direct comparison of the clonogenic surviving fractions after individual and combination therapy of the cell line 134B-TK, for example Fig. 5.10, indicated possible dose dependent supra-additive effect at the low surviving fractions (i.e. at higher concentration of GCV/ [^{123}I]FIAU) of all combination schedules in this cell line. However, this comparison is not reliable, as it does not consider the sigmoidicity of the dose-effect curves and the exclusivity of drug effects. The subsequently generated CI values have confirmed synergistic drug interaction between the two agents in the treatment of 143B-TK by all combination schedules. However, exposure to the smaller

doses of all combination schedules resulted in CI values greater than 1, indicating antagonism or infra-additive effect.

Unlike the results obtained in 143B-TK cells, the CI values of the prostate cancer cell line DU145-TK were generally higher than 1, indicating antagonism. One exception was the high toxicity concentration range of the treatment schedule of GCV before FIAU (Fig. 5.7). Variations in cell lines's response to combination therapies have been reported previously in several *in vitro* and *in vivo* studies (Aoe et al., 1999; Engblom et al., 1999; Kano et al., 1999; Topaly et al., 2002); (Bollig et al., 2005). One possible explanation of the antagonistic interaction observed in the prostate cancer cell line is the theoretical competitive mutual inhibition between FIAU and GCV for HSV1-TK phosphorylation, which is a critical step for their intracellular retention and subsequent cytotoxicity.

The dose dependent synergism which was observed with the cell line 143B-TK might indicate enhancement of tumour cell radiosensitivity to [¹²³I]FIAU. It has been demonstrated in extensive series of preclinical studies that the HSV1-TK/GCV suicide gene system is a potent tumour cell radiosensitiser (e.g. (Elshami et al., 1997; Kim et al., 1994a; Kim et al., 1995; Vlachaki et al., 2001). A possible explanation for this radiosensitivity enhancement is that HSV1-TK system inhibits DNA sub-lethal repair after radiation damage (Kim et al., 1995). Firstly, GCV inhibits DNA polymerase α , the enzyme that has been implicated with the repair of potentially lethal damage. Secondly, phosphorylated GCV is incorporated into the DNA, making fraudulent DNA, which is more susceptible to strand breakage by radiation.

The limitation of synergism to the higher doses of drug combinations (dose-dependent synergism) is one of the recognised phenomena in the preclinical assessment of combination therapy in oncology (e.g. (Johnston et al., 2003). We attempted to explain

this and the difference between cell lines response by the analysis of cell cycle distribution.

The cell line DU145-TK response to the combination schedule of FIAU-before-GCV showed antagonistic effect throughout the whole concentration range. In contrast, the cell line 143B-TK exhibited different interaction with synergistic effect at the high toxicity concentration range and additive effect at the low toxicity concentration range. Cell cycle analysis showed negligible changes after 4 h exposure to [¹²³I]FIAU in both cell lines to a similar extent which does not explain their different response to this combination schedule. However, the similar response of both cell lines to the GCV-before-FIAU schedule coincided with a similar cell cycle shift to S and G₂/M phases as shown in Fig. 5.9. Previous *in vitro* work has shown that HSV1-TK/GCV treatment leads to a gradual increase in the proportion of cells in S phase in keeping with the mechanism of action of the activated GCV as an anti-metabolite (McNeish et al., 2001). Although the evidence of synergism in this study was dose- and schedule-limited it is worth supporting these findings by an *in vivo* work in tumour xenografts or orthotopic prostate cancer models. Another issue that might be addressed is the use of other FIAU radioiodine conjugates such as the Auger electron emitter ¹²⁵I and the α -particle emitter ¹³¹I. One advantage of these radionuclides is their longer half-life. Furthermore, enhanced cell-kill might be achieved by the bystander and cross-fire effect of ¹³¹I.

Chapter 6 : Conclusion and future work

6.1 Conclusion

The main hypothesis of this study was that there is a therapeutic benefit of delivering radiolabelled FIAU, alone or in combination with GCV, to prostate cancer tumour cells bearing the HSV-*tk* gene. As in other novel protocols of gene therapy, the process of testing this hypothesis requires a series of preclinical laboratory investigations. We have, in this thesis, presented the results of our *in vitro* experimental work on this strategy, which we believe is supportive of this hypothesis.

In our study, the initial HSV1-*tk* gene transfer into tumour cells using a plasmid vector and the cationic lipid effectene transfection reagent was shown to be highly efficient method which agrees with previous studies, e.g. (Uchida et al., 2002). This was confirmed by the transfection efficiency experiments. Despite a large variation in GCV sensitivity, the three cell lines used in this study exhibited comparable degrees of [¹²³I]FIAU uptake and sensitivity. The difference in GCV sensitivity enhancement by the thymidine kinase gene transfer was attributed to the variations amongst the cell lines rather than to the type of the plasmid vector or the transfection efficiencies as it was seen in previous studies, e.g. (Nagy et al., 2000).

This study has illustrated that [¹²³I]FIAU was cytotoxic to tumour cells expressing the HSV1-*tk* gene. This cytotoxicity, which was evaluated by clonogenic assay, was dose-dependent and it was enhanced by the prolongation of the incubation period. There was a strong correlation between the proliferation rate and the cellular uptake and sensitivity of tumour cells bearing the HSV1-*tk* gene to radiolabelled FIAU. This confirms the notion that cellular proliferation is essential in this treatment modality which will work more efficiently for rapidly growing tumours. Moreover, there will be an advantage of

sparing the normal slow growing tissues. This is in agreement with the findings of previous studies (Neshasteh-Riz et al., 1998); (Audouy et al., 1999).

Optimum tumour cell kill was achieved when the monolayers of the TK-positive clones of the DU145 and 143B cell lines were treated with [¹²³I]FIAU for an incubation period equivalent to the cellular doubling time. This will probably have an implication on the clinical application of this strategy related to the schedule of administration of [¹²³I]FIAU.

The treatment of the spheroid models of the TK-positive clones of the three cell lines failed to elicit a greater cell kill than that observed in the monolayers. For example, the IC₅₀ value for the monolayers the cell line DU145-TK was 0.89 (±0.05) MBq/ ml compared with 0.91 (±0.06) MBq/ ml for the spheroids (*P* > 0.05). This was anticipated according to the results obtained from previous work by our group using another thymidine analogue labelled with ¹²³I (Neshasteh-Riz et al., 1998). As discussed previously, we attributed this to the ultra-short range of the Auger electron emitted as a result of ¹²³I decay.

It has been shown previously that suicide gene therapy using HSV1-*tk* and GCV in conjunction with spheroid models revealed even lower cellular toxicity when compared with the effect on monolayers of tumour cell lines (Finocchiaro et al., 2004). This was suggested to be secondary to compromised prodrug penetration and/ or higher fraction of quiescent cells in the spheroids that would be naturally resistant to this cell cycle-dependent system.

The final part of this study was a trial to amalgamate the strategy of targeted radiation cytototherapy with suicide gene therapy protocol using the transgene encoding the HSV1-TK enzyme. The combination treatment of the cell line 143B-TK revealed dose-dependent synergistic interaction following all treatment schedules. The most favourable

combination schedule of the prostate cancer cell line DU145-TK was the GCV-before-FIAU schedule which resulted in a dose-dependent additive effect. Both cell lines, however, exhibited an antagonistic combination effect at the low toxicity range of concentrations of the two agents. Although the two cell lines responded differently to this combination therapy, there was an evidence of synergism in one cell line that warrants further investigation in other cell lines and animal models.

We have also investigated the impact of the individual treatment with GCV or [¹²³I]FIAU on cell cycle progression in TK-positive clones of the two cell lines DU-145 and 143B-TK. The treatment of the TK-positive cell lines with [¹²³I]FIAU for 4 h and GCV for 72 h caused no significant cell cycle redistribution. A significant finding was the cell cycle arrest at G2/M-phase following the exposure to [¹²³I]FIAU for a period equal to the doubling time of the respective cell lines. Due to the fact that tumour cells are most radiosensitive in the G2-phase, one of the implications of this cell cycle arrest would be the potential benefit of scheduled combination of this strategy with EBRT for the treatment of prostate cancer.

6.2 Future work

Contrary to the traditional pharmaceutical preclinical drug research, experimental gene therapy involves at least two extra steps such as gene transfer and the assessment of its long term sequels. This is one of the hurdles which are delaying the progress of numerous strategies of gene therapy.

The main part of our future work will include testing the effect of radiolabeled FIAU on other cell lines of prostate cancer and other tumours both with and without the viral *tk* gene expression. Further work is also required to improve the transfection efficiency of the viral *tk* gene. In terms of prostate cancer, this will involve trying different tissue- and tumour-specific promoters such as the PSA and the telomerase promoters. Furthermore, the results obtained from the spheroid experiments were disappointing and, therefore, more work is needed using longer incubation periods and utilising FIAU conjugated with different isotopes of the radioactive iodine such as the β -particle emitter ^{131}I .

In concordance with the ethics of preclinical research, this experimental therapeutic approach should be tested in animals before any clinical trial could be approved.

To support the findings of our study, the first step of the experimental animal work would aim to confirm the therapeutic potential of the radiolabelled FIAU for tumours transfected with the HSV1-*tk* gene. Most animal studies are currently performed using transplanted tumours produced by the subcutaneous or intramuscular implantation of cancer cell lines (xenografts). Subcutaneous heterotopic graft models are often used because they are relatively easy to generate. Unfortunately, this approach completely ignores the histological and anatomical specificity of the organ under investigation. Consequently, although they are useful in a limited fashion, these studies do not adequately serve as preclinical trials of human gene therapy (Shimizu et al., 2001). It

will be advantageous; therefore, to investigate this novel approach of gene therapy using orthotopic tumour models such as the inoculation of cancer cells into the prostate gland of the experimental animal (Freytag et al., 2002b).

A potential model could initially involve *ex vivo* transfer of the HSV1-*tk* gene into a cell line derived from prostate cancer and then orthotopic inoculation of these transfected cells into the prostate gland of a number of rats or mice. The ability of these cells to form orthotopic tumour could be assessed by *in situ tk* gene expression imaging using systemic administration of radiolabelled FIAU and PET (Tjuvajev et al., 2002) or SPECT scanning (Gambhir et al., 2000)

Alternatively, these orthotopic prostate tumours could be detected using small animal MRI scan (Abdulkadir et al., 2001) or by monitoring serum PSA (Freytag et al., 2002b). The other model of orthotopic prostate cancer has an additional step of *in vivo* transfer of the HSV1-*tk* gene into previously prepared TK-negative orthotopic prostate tumours e.g. (Zhang et al., 2002). This model recapitulates better the real clinical scenario when treating organ-confined human prostate cancer with this strategy.

In addition, rodent prostate carcinoma models have been developed, which could also serve as preclinical models for this strategy. Methods to develop these rodent models include hormone treatment (Noble, 1977), transgenic prostate-specific oncogene expression (Greenberg et al., 1995), and knockout of tumour suppressor genes (Podsypanina et al., 1999).

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