CELL BIOLOGICAL RESPONSES OF PROSTATIC TUMOUR CELL LINES TO IRRADIATION AND ANTICANCER DRUGS.

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any University for a degree.

Signature   Date

ABSTRACT

The "classic" prostate cell lines, DU145, PC-3 and LNCaP, have served as a valuable cell biological model for research into prostate cancer. However, their relevance may be limited because they derive from metastatic, and not from primary normal and tumour epithelium. The cell lines (1532T, 1535T, 1542T, 1542N and BPH-1) have been derived from primary benign and malignant human tumour prostate epithelium and may be more representative. Using these cell lines I have examined the role of basic cell damage responses (repair, checkpoint activation, apoptosis and associated signalling proteins, and the influence of androgen status) in cell inactivation, and its relevance to treatment.

Numerous studies have suggested that loss of p53 function leads to resistance to chemotherapeutic agents and irradiation. It is shown here that the p53-inactive cell lines are, in fact, the most sensitive to chemotherapeutic agents such as etoposide, vinblastine and estramustine, whilst the p53 wild-type cell line, LNCaP, is the most radiosensitive. Notwithstanding the effects of p53 degradation by the HPV-16 E6 viral protein, the results on chemosensitivity raises the possibility that different chemotherapeutic agents may have different p53-dependent effects in different tumour cells.

Androgen deprivation is demonstrated to sensitise prostate cancer cells to chemotherapeutic agents and it is shown that the hormone independent cell lines are the most chemosensitive. The LNCaP cell line displayed an increased resistance to apoptosis induced by etoposide and gamma irradiation, suggesting that androgens are capable of protection against both these DNA damaging agents.

The major factors determining radiosensitivity in human tumour cell lines are known to be DNA double-strand break (dsb) induction and repair. In the prostate cell lines I find that cellular radiosensitivity correlates with the number of DNA double-strand breaks measured within 2 hours of irradiation, and that the more radioresistant cell lines show better repair competence. Conclusions as to the influence of androgen

dependence on radiosensitivity and repair are not possible at this stage since only the LNCaP cell line was androgen sensitive. The fact that the 2 hour repair period can separate radiosensitive from radioresistant cells in 2 groups of human tumour cell lines highlights the role of non-homologous end-joining repair. This has implications for therapy, and is consistent with the clinical observation that prostate tumours can be successfully controlled by low dose rate-brachytherapy.

To evaluate the role of apoptosis, cells were exposed to TD_{50} concentrations of chemotherapeutic drugs, and ^{60}Co γ-irradiation. Apoptosis was found to be low, overall, and ranged from 0.1% - 12.1%, 3.0% - 6.0% and 0.1% - 8.5% for etoposide, estramustine and vinblastine, respectively. The percentage of cells undergoing drug-induced apoptosis was, on average, higher in the tumour cell lines than in the normal cell lines. Gamma irradiation-induced apoptosis levels ranged from 1.3% - 7%. The LNCaP cell line yielded the lowest percentage of apoptotic cells after exposure. The 1532T cell line yielded the highest percentage of apoptotic cells after exposure. Apoptotic propensity did not rank the cell lines according to their radiosensitivity.

Immunoblotting demonstrated that the apoptosis-associated proteins, bax and bcl-2, are expressed at a basal level in all the cell lines tested, but no increase was detected after exposure to TD_{50} doses of etoposide, vinblastine and estramustine. The ratio of bax and bcl-2 also was not altered by DNA damage.

No evidence was found that a correlation may exist between reproductive cell death and the expression of genes which control apoptosis. My results show that apoptosis is not a major mechanism of drug- or radiation-induced cell death in prostate cell lines.

In conclusion, loss of p53 function and loss of androgen dependence was not found to be correlated with resistance of tumours to chemotherapeutic drugs. Cellular radiosensitivity was found to be correlated with the number of DNA double-strand breaks remaining after 2 hours of repair. The more radioresistant cell lines showed better repair competence. Apoptosis and genes affecting apoptosis, such as p53 and members of the bcl-2 family, do not seem to contribute significantly to the sensitivity of prostate cancer cells to anticancer drugs and irradiation.

ABSTRAK

Die klassieke prostaat sellyne, DU145, PC-3 en LNCaP, het 'n waardevolle bydrae gemaak in die sel biologiese model in prostaat kanker. Die toepaslikheid daarvan mag egter beperk wees, aangesien hierdie sellyne afkomstig is van metastatiese, en nie van primêr normale en tumor epiteel nie. Die sellyne 1532T, 1535T, 1542T, 1542N en BPH-1 is afkomstig van primêre benigne en maligne menslike prostaat tumor epiteel en mag moontlik meer verteenwoordigend wees. Deur van hierdie sellyne gebruik te maak, is die rol ondersoek van die reaksie op basiese selskade (d.w.s. herstel, beheerpunt aktivering, apoptose en verwante sein proteïene, en die invloed van androgeen status) tydens die proses van sel inaktivering, asook die toepaslikheid ten opsigte van behandeling.

Volgens verskeie studies lei die verlies aan p53 funksie tot weerstandigheid teen chemoterapeutiese middels en bestraling. Die resultate van hierdie studie toon dat die p53-onaktiewe sellyne egter die sensitiefste is vir chemoterapeutiese middels, soos etoposied, vinblastien en estramustien, terwyl die p53 natuurlike-tipe sellyn, LNCaP, die meeste radiosensitief is. Ten spyte van die invloed van p53 afbraak deur die HPV-16 E6 virale proteïen, dui die resultate van chemosensitiwiteit op die moontlikheid dat verskillende chemoterapeutiese middels verskillende p53-afhanklike effekte op verskillende tumorselle mag hê.

Dit is bewys dat onttrekking van androgeen prostaat kankerselle sensitiseer teen chemoterapeutiese middels en dat hormoon-onafhanklike sellyne die hoogste chemosensitiwiteit vertoon. Die LNCaP sellyn vertoon 'n verhoogde weerstandigheid teen apoptose wat deur etoposied en γ-bestraling geïnduseer is, wat 'n aanduiding is dat androgene beskerming kan bied teen beide hierdie DNA beskadigingsfaktore.

Die belangrikste faktore wat die radiosensitiwiteit in menslike tumorselle bepaal, is bekend dat dit die dubbelbande van DNA verbreek en herstel. Hierdie studie het aangetoon dat in prostaat sellyne die sellulêre radiosensitiwiteit korreleer met die aantal DNA dubbelband verbrekings binne 2 uur na bestraling, en dat die meer radioweerstandige sellyne beter herstelvermoë vertoon. Gevolgtrekkings oor die

invloed van androgeen se afhanklikheid van radiosensitiwiteit en herstel kan egter nie op hierdie stadium gemaak word nie, aangesien slegs die LNCaP sellyn androgeen-afhanklik was. Die feit dat die 2 uur herstelperiode 'n skeiding kan maak tussen radiosensitiewe en radioweerstandige selle in twee groepe menslike tumor sellyne, onderstreep die rol van herstel van nie-homoloë endverbindings. Dit hou implikasies in vir terapie, en stem ooreen met die kliniese waarnemings dat prostaat tumore suksesvol gekontroleer kan word deur lae intensiteit dosis bragiterapie.

Ten einde die rol van apoptose te ondersoek, is selle blootgestel aan TD$_{50}$ konsentrasies chemoterapeutiese middels, asook ^{60}Co γ-bestraling. Apoptose was oor die algemeen laag, en het gestrek van 0.1% tot 12.1%, 3.0% tot 6.0% en 0.1% tot 8.5% vir etoposied, estramustien en vinblastien onderskeidelik. Die persentasie selle wat middel geïnduseerde apoptose ondergaan het, was gemiddeld hoër in tumor sellyne as in normale sellyne. Die waardes van apoptose geïnduseer deur γ-bestraling het gewissel van 1.3% tot 7.0%. Die LNCaP sellyn het die laagste persentasie apoptotiese selle na bestraling gelewer, terwyl die 1532T sellyn die hoogste persentasie gelewer het. Die volgorde van die radiosensitiwiteit van die sellyne was nie waarneembaar in hulle geneigdheid tot apoptose nie. Immunoblots het aangetoon dat die apoptose-geassosieerde proteïene, bax en bcl-2, uitgeskei word teen 'n basisvlak in al die sellyne wat getoets is, maar dat geen verhoogde uitskeiding waarneembaar was na blootstelling aan TD$_{50}$ dosisse etoposied, vinblastien en estramustien nie. Die verhouding van bax en bcl-2 is ook nie beïnvloed deur DNA beskadiging nie.

Dit blyk daarom dus onwaarskynlik dat daar 'n korrelasie bestaan tussen reproduktiewe seldood en die uitskeiding van gene wat apoptose beheer. Die resultate dui daarop dat apoptose nie 'n belangrike meganisme vir middel- of bestralingsgeïnduseerde seldood in prostaat sellyne is nie.

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DEDICATION

JOHN PETER LOWE

A gentleman, and a gentle man

For the journey is done and the summit attained,
And the barriers fall,
Though a battle's to fight ere the guerdon be gained,
The reward of it all.

Robert Browning

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LIST OF ABBREVIATIONS

OD	Optical density
PI	Propidium iodide
FITC	Fluorescein isothiocyanate
PBS	Phosphate buffered saline
PE	Plating efficiency
FBS	Foetal bovine serum
FACS	Fluorescence Activated Cell Sorter
csFBS	Charcoal-stripped foetal bovine serum
TD	Toxic dose
SDS	Sodium dodecyl sulphate
DHT	5α - dihydrotestosterone
SF2	Survival fraction at 2Gy
SSD	Source-to-sample distance
BSA	Bovine serum albumin
AUC	Area under the dose response curve
IgG	Immunoglobulin G
CFGE	Constant-field gel electrophoresis
NHEJ	Non-homologous end-joining
HR	Homologous recombination

$\overline{D}$	Mean inactivation dose after ^{60}Co γ-irradiation
dsb	Double-strand break
ssb	Single-strand break
HPV-16	Human papilloma virus serotype 16
SV40	Simian virus 40
EDTA	Ethylene diamine tetra-acetic acid
TBE	Tris buffered EDTA
PVDF	Polyvinylidine difluoride
TBST	Tris buffered saline-Tween 20
Gy	Gray (1Gy = 100 rad)
DMEM	Dulbecco's modification of Minimum Essential Medium
α	Linear coefficient of inactivation after ^{60}Co γ-irradiation
β	Quadratic coefficient of inactivation after ^{60}Co γ-irradiation
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
Temed	N,N,N',N'-Tetramethylethylenediamine

CHAPTER 1

INTRODUCTION

Prostate cancer – the clinical problem

Prostate cancer is the second most common cancer in South African men, with an estimated annual incidence of 19.1 per 100 000 men (Sitas 1994). However, this incidence rate is probably deceptively low owing to incomplete reporting of cases. Comparison of the incidence of prostate cancer in the different racial groups shows that it is the second most common malignancy in Caucasian, African, and so-called Coloured (Mixed race), and the fourth most common malignancy in Asian men in South Africa (Sitas 1994). Prostate cancer is the most frequently diagnosed malignancy and the second leading cause of cancer-related deaths among men in Western industrialised countries (Miyake *et al* 2001).

Prostate cancer is often found incidentally during transurethral resection of the prostate, which is performed to relieve obstruction normally associated with benign prostatic hyperplasia. Since the incidence of prostate cancer increases as a function of age, there is a misconception that it is a disease of the very elderly. However, approximately 20% of prostate cancers occur in men under the age of 65 years (Boring *et al* 1994). While the majority of prostate cancer cases occur in men over the age of 65 years, the impact of the disease is still significant.

The clinical course of the disease is difficult to predict. About 50% of men with prostate cancer have clinically advanced (ie. extra-prostatic) disease at the time of initial diagnosis. 15% of those with organ-confined disease actually have micrometastatic disease at the time of surgery (Carter and Coffey 1988). In some patients, prostatic cancer metastasises rapidly, killing the patient within a year of diagnosis. Other men survive untreated for many years with localised disease, with no clinically detectable metastases (Johannson 1980). If completely localised (ie. within the prostatic capsule) prostatic cancer can be cured by surgery alone (ie. radical

prostatectomy) (Walsh and Jewett 1980). However, when the disease is extra-prostatic, it is usually fatal and there is no curative treatment.

Treatment of prostate cancer

The traditional goal in the treatment of genitourinary cancer has been to maximise patient survival. Recent advances in urologic oncology have had a positive impact on prognosis, permitting many patients to live significantly longer with their disease. When critically evaluating interventions in these patients it must be remembered that cancer affects both quantity and quality of life. The goal of treatment (curative, palliative), the treatment-associated morbidity, and not least, patient preference must be considered when therapy decisions are made.

Complications after treatment of prostate cancer are well documented. The major concerns of most patients are urinary incontinence and impotence (deKernion *et al* 1998).

Until effective systemic treatments can be developed, the best hope of decreasing the mortality rate from prostate cancer lies in curative treatment while the tumour is still organ-confined. The treatment choices for localised prostate cancer are limited and include either radiotherapy or radical prostatectomy. Neither of these treatment choices has demonstrated clear superiority in terms of long-term cure or disease-free survival (Hartford and Zietman 1996). Each option has distinct advantages and disadvantages, and the final treatment decision is largely based on the preference of the individual.

Radiation Therapy

The first and by far the most useful radiation modality currently used in the treatment of prostate cancer is external beam radiation, using mostly high-energy photons. For patients with true early-stage prostate cancer (stage T1-T2, and a prostate specific antigen (PSA) level less than 15ng/ml) external beam radiation therapy offers a good chance of durable tumour control or eradication (Porter and Hart 1999). Among such patients there are no survival differences between those treated with irradiation and those treated with surgery. Men who are more than 65 years old have little, if

anything, to gain from surgical management. Currently, treatment recommendations are that younger men (less than 60 years) be considered for surgery first, although external beam radiation remains an excellent alternative. The relative morbidities of the two alternatives must be kept in mind when discussing treatment issues with patients: long-term impotence or incontinence may be of great concern for a younger patient.

For patients with locally advanced cancer (T3-T4), and a PSA level greater than 15ng/ml) radiation therapy offers effective and durable palliation, but little chance for long-term cure (Hartford and Zietman 1996).

Chemotherapy

Most of the mortality and morbidity from prostate cancer occurs in the setting of metastatic disease, particularly in hormone-refractory disease. Therapy is primarily palliative and aimed at delaying the onset and progression of symptoms. Once the disease becomes resistant to hormonal suppression, the median survival is only 12-18 months (Raghavan 1988, Crawford *et al* 1989, Greenlee *et al* 2000). Options for therapy include secondary hormonal therapy, chemotherapy, and experimental therapies.

The prevailing opinion for the past two decades that chemotherapy is not very effective in advanced prostate cancer could be due to: a lack of properly designed clinical trials, the administration of chemotherapy when the tumour burden is high, the fact that chemotherapeutic drugs currently available in clinical use preferentially kill rapidly proliferating cancer cells, and the significant toxicity associated with many of the drugs (CF Heyns, personal communication).

Chemotherapy should be considered for patients in whom the potential benefits of palliation outweigh the risks of toxicity (Sternberg and Ianari 1996).

The case for chemotherapy

The historical negative view for chemotherapy (which stemmed from poor efficacy) has now been replaced by a moderate enthusiasm. The last decade indeed has

witnessed a new desire to investigate the potential benefits of chemotherapy. This has been fostered by the development of several new drug regimens to accomplish palliative responses in hormone-refractory prostate cancer.

The ability to slow and even reverse the growth of far-advanced disease raises the possibility that the application of this therapy earlier in the disease will have a more significant impact on mortality.

The emergence of prostate specific antigen (PSA) as a surrogate marker of disease burden and response of advanced prostate cancer has revolutionised the study of chemotherapy in hormone-refractory disease. Research to investigate the wider role of chemotherapy prior to prostatectomy or radiation therapy is currently underway.

Estramustine phosphate (EMP), an agent that combines estradiol with nitrogen mustard, has been shown to have activity against prostate cancer cells independent of its hormone moiety and its alkylating moiety. Originally synthesised to allow selective delivery of the alkylating agent into oestrogen receptor-positive cancer cells, it has been shown to have little alkylating activity and acts by binding to microtubules and the nuclear matrix. The significance of the interaction is unknown, however, and it was postulated that EMP may bind to the nuclear matrix and interfere with the DNA replication process (Pienta and Lehr 1993). New evidence suggests that estramustine functions as a microtubular poison and is synergistic with multiple cytotoxic agents *in vitro* and *in vivo* (Benson and Hartley-Asp 1990).

It is theorised that estramustine in combination with other microtubule inhibitors (vinblastine, for example), with different sites of action, might result in an additive or synergistic anti-tumour effect. Van Belle *et al* (1988) showed a greater than 50% decline in PSA levels in 43% of patients treated with this combination in three subsequent clinical trials. Estramustine, too, has been combined with etoposide, a topoisomerase II inhibitor. *In vitro* and *in vivo* prostate cancer growth is synergistically inhibited by this combination, by interaction at the nuclear matrix (Pienta and Lehr 1993). A decline in the PSA level of more than 50% occurred in 52% of the phase II trial patients.

In assessing newer chemotherapy agents in the treatment of prostate cancer, it is important to recall that the vast majority of the agents that have subsequently been shown to have significant activity in combinations had little activity as single agents.

Androgen independence

Prostate cancer is a heterogeneous disease (Isaacs and Coffey 1981, Logothetis *et al* 1994). Cell lines and animal models provide important insights into the biological basis for this heterogeneity, although our understanding of the mechanism of androgen independence remains incomplete.

Initially, the growth of the majority of prostate cancers is androgen dependent. Consequently, prostate cancer patients benefit temporarily from androgen-suppressing therapies, either by surgical or chemical castration, by anti-androgens, or by a combination of these. After an initial androgen dependent growth phase, most prostate tumours enter an androgen independent stage. The molecular basis for this switch from androgen dependent to androgen independent growth is largely unknown.

In the Dunning rat model engrafted prostate tumours are composed of a mixture of androgen dependent and androgen independent cells. With castration androgen dependent cells die and the tumour stops growing. However, androgen independent cell growth occurs simultaneously and ultimately a palpable mass develops. Thus, androgen ablation provides a selective advantage to androgen independent cells that grow and eventually comprise the abundance of the tumour (Isaacs and Coffey 1981). This scenario has been confirmed in other systems, including the human prostate cancer cell line, LNCaP (Horoszewicz *et al* 1983).

Alteration in normal androgen signalling probably has a central role in the pathogenesis of androgen independent prostate cancer. Mutations in the androgen receptor gene, in contrast to the oestrogen receptor gene in breast cancer, theoretically should confer phenotypic changes more readily since the androgen receptor gene is x-linked and, therefore, requires only one allele to be altered (Taplin *et al* 1995). Androgen independence, in theory, may be mediated through mutations of the androgen receptor gene that alter expression of androgen receptor or its ligand sensitivity. Several groups have described various mutations in the steroid binding

domain of the androgen receptor in patients with androgen independent prostate cancer (Taplin *et al* 1995, Elo *et al* 1995). Other investigators have found androgen receptor missense mutations in tumours from patients with androgen independent prostate cancer (Visakorpi *et al* 1995, Zhao *et al* 1999), although the frequency of these mutations is debated. This variable mutation frequency may reflect, in part, different patient populations, the methods used to detect mutations, or tumours from primary versus metastatic sites. Most studies in patients treated with androgen ablation monotherapy have found a low frequency of androgen receptor mutations. Most mutations were found in patients treated with an androgen receptor antagonist (flutamide) in conjunction with androgen ablation (Balk 2002). This finding suggests that flutamide treatment is selecting for tumour cells with androgen receptor mutations. The mutation found most frequently in the flutamide-treated patients (codon 877 threonine to alanine) was identical to the mutation identified initially in the LNCaP cell line (Veldscholte *et al* 1990).

In the early stages of prostate carcinogenesis, cells retain some degree of androgen dependence and respond to androgen ablation therapy. Disease progression is associated with mutations and produces clones of autonomous cells and autocrine growth-promoting factors which are unresponsive to androgen withdrawal (Lee 1997). The proliferation of such clones may be promoted by the autocrine production of transforming growth factor-alpha (TGF-α) through the epidermal growth factor receptor (EGF-R). Enhanced growth factor production through oncogene activation, dysfunctional growth factor receptor expression, and ultimate loss of growth suppressors such as p53 and retinoblastoma (Rb) are probably responsible for the androgen independent progression of prostatic cancer (Griffiths and Morton 1999).

The role of p53 in damage responses

Tumour suppressor genes play a fundamental role in cell division and growth control. When these genes fail to function properly, deregulated growth -- a defining feature of cancer cells -- ensues. The p53 tumour suppressor, first described in 1979 (Lane and Crawford), has emerged as the most commonly altered gene and growth suppressing pathway in human cancer.

The p53 tumour suppressor protein is often described as the "guardian of the genome" because it is a critical component of the cell in its response to genotoxic stress ie. DNA damage, hypoxia, etc. (Figure 1). P53 maintains genomic integrity in part by arresting cell cycle progression or by inducing apoptosis (Levine 1997). Consistent with this view, about 50% of primary human tumours carry mutations in the p53 gene (Greenblatt *et al* 1994). The mechanism by which p53 is activated by cellular stress has recently been shown to depend on ATM proteins which associate with p53 in the absence of DNA damage. Following double-strand break formation, the complex dissociates when they phosphorylate each other at serine position 1981 in the protein (Bakkenist and Kastan 2003).

Wild-type p53 plays a role in the induction of G_1 arrest, DNA repair and apoptosis following DNA damage (Lee and Bernstein 1995). The p53-mediated G_1 arrest results from the transactivation of several cellular molecules, in particular p21 and gadd45 (Hwang and Muschel 1998). P21 inhibits CDK/cyclin activity which indirectly suppresses the expression of the transcription factor E2F, required for the expression of S-phase-specific genes. This causes a G_1 arrest (Gartel *et al* 1996). The high levels of p21 and gadd45 increase repair mediated by proliferating cell nuclear antigen (PCNA) and DNA excision (Prosperi 1997). The mdm2 gene is positively regulated by p53, and inhibits p53 function (Wetzel and Berberich 1998). P53 activation also leads to the synthesis of cyclin G, which is thought to be involved in G_2 arrest (Shimitzu *et al* 1998). P53 induces apoptosis through pathways which are not yet fully understood, but is thought to involve activation of the ICE proteases and the bax gene which acts as a positive regulator (Enoch and Norbury 1995). Bcl-2 inhibits apoptosis (Hickman 1998).

Since the p53 protein is involved in all known damage responses and might also play a role in G_2 arrest (this is still not well-defined), the cellular p53 status plays a crucial role in cell survival. The downstream effects of p53 have lead to contradictory hypotheses regarding the influence of p53 status on radiosensitivity (Bristow *et al* 1996). For example, wild-type p53 could facilitate apoptosis leading to increased cell death, but it could also induce a G_1 and/or G_2 arrest and increase DNA repair leading to increased cell survival. Many contradictory reports have been published in this regard (for review see Morgan and Kastan 1997) which may be explained by the fact

that p53-dependent apoptosis and G_1 arrest followed by DNA repair and cellular recovery appears to be controlled by different downstream pathways and that the activity of these pathways is cell-type specific. The fact that a large number of tumour types exhibit mutated p53 genes distinguishes them from normal cells. It may be possible to exploit this to kill tumour cells. One example is the preferential chemo- and radiosensitisation of p53 mutant tumour cell lines by radiosensitising agents which abrogate the G_2 cell cycle block (Binder *et al* 2000, Serafin *et al* 2001).
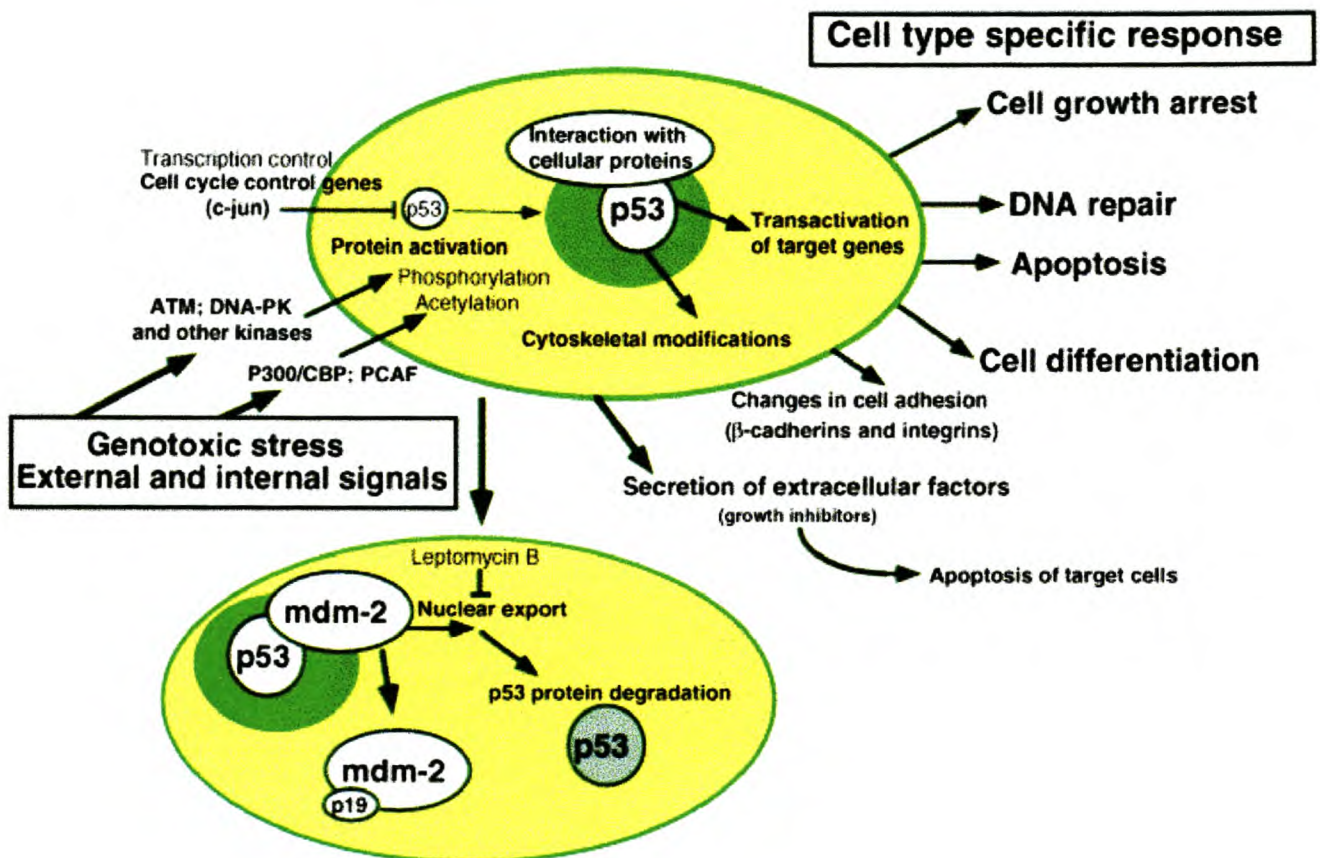
Figure 1: p53 is activated by several types of stress and induces the expression of several proteins that are involved in the inhibition of cell proliferation or in promoting cell death by apoptosis. In tumour cells, the growth-arresting and apoptotic effects of p53 might be lost owing to the mutation of genes downstream of p53, to mutations in the p53 gene, or to mutations or alterations in the levels of proteins that modulate the p53 function.

Molecular response mechanisms

Apoptosis

Apoptosis is a central mechanism in tissue homeostasis ensuring that cell loss always equals cell gain from mitosis. Disruption of the fine balance between cell proliferation and cell death is thought to be an important step in the complex process of carcinogenesis (Berges *et al* 1995).

Apoptosis has several distinct features which distinguish it from necrosis (Searle *et al* 1982). These are chromatin condensation, cell shrinkage, membrane blebbing and DNA degradation at internucleosomal linkages (Wyllie *et al* 1981). This results in the characteristic nucleosomal DNA repeat ladder evident in DNA electrophoretic gels. Apoptotic cells are usually scattered throughout tissue rather than occurring in a large block of contiguous cells as found in necrosis (Searle *et al* 1982). In contrast to necrosis, inflammation is absent in apoptosis. Unlike necrosis, apoptosis arises from activation of a specific genetic program. It has been described as "death from the inside out". In the cell, apoptosis is initiated by a cascade of highly specialised sequences of protein and DNA cleavage. Protein cleavage results from activation of a family of cysteine proteases or "caspases", while DNA is cleaved by endonucleases (Peter and Krammer 1998). The activation of these processes can only start if the cell has sensed damage and signalled this to the appropriate cell machinery. A number of tumour suppressor genes such as p53, and oncogenes such as *myc* and *bcl-2*, have been implicated in the recognition and signalling process (Konopleva *et al* 1999). Intricate pathways leading to apoptotic death have been identified, eg. the ceramide pathway (Venable *et al* 1995, Mizushima *et al* 1996), the stress-activated protein/jun kinase (SAPK/JNK) cascade (Kasibhatla *et al* 1998), and the mitogen-activated protein kinase/extracellular-signal regulated (MAPK/ERK) pathway (Blagosklonny 1998). A simplified diagram describing the basic apoptotic pathways is given in Figure 2.

Several other molecules like bcl-2, bax, and other pro- and anti-apoptotic molecules determine the survival "threshold" of a certain cell type (Reed 1995) and have been identified in androgen independent prostate cell lines and tissues. Androgen

independent prostate cells maintain the ability to undergo apoptosis since the apoptotic program appears to be functional, if not intact and complete (Denmeade *et al* 1996); thus, it appears that androgen withdrawal fails to initiate the apoptotic pathway. Possible reasons for this failure could be the increased expression of genes associated with enhanced cellular survival such as bcl-2, or p53 mutations involved in triggering apoptosis in response to injury or DNA damage (Colombel *et al* 1993). The anti-apoptotic protein, bcl-2, seems to be important to the development of the androgen resistant phenotype and to the resistance to chemotherapeutic agents (Raffo *et al* 1997). This hypothesis has gained support from recent observations that PC-3, a p53 null, androgen independent human prostate cancer cell line, can be triggered to undergo apoptosis upon treatment with several conventional chemotherapeutic drugs including cisplatin, etoposide and vincristine, and camptothecin (Borner *et al* 1995).

Normal human prostatic secretory cells do not express the bcl-2 protein. Hormone refractory prostate cancer tissue derived from hormone-treated patients show a strong bcl-2 expression suggesting that the increased expression of this protein provides prostate cancer cells with the ability to survive outside of the hormonal milieu (Berchem *et al* 1995). Interestingly, androgens have been shown to induce expression of bcl-2 protein in hormone sensitive LNCaP cells, which may represent one of the mechanisms whereby hormone dependent prostatic cancer cells evolve into androgen independent cells.

On the other hand, downregulation of bcl-2 expression with gene-specific antisense oligos has been shown to abolish the bcl-2-conferred resistance to apoptosis induction (Berchem *et al* 1995).

The p53 tumour suppressor gene is the most frequently mutated gene found in multiple human malignant tumours, including prostate cancers. Several studies have shown p53 mutations to be relatively rare in prostate cancer, in general, but to be much more frequent in advanced tumours. Mutations of the naturally present wild-type p53 permit cells with altered genomic composition to maintain proliferation which in turn can result in the acquisition of additional genetic abnormalities (Sakr and Grignon 1997).

Whether p53 is a requirement of prostate cancer cells to undergo apoptosis is still unclear. The fact that androgen ablation-induced apoptosis does not appear to involve p53 and that multiple chemical entities induce apoptotic death of p53 null PC-3 cells (Borner *et al* 1995) suggests that p53 may not be essential for the apoptotic response to occur in these situations (Berges *et al* 1993). However, since p53 has an important role in introducing the G_0/G_1 checkpoint, attenuation or loss of p53 activity after DNA damage could represent an early event in prostate oncogenesis, leading to an increased probability of a cell accumulating the genetic alterations necessary for transformation.

It is widely suggested that apoptotic propensity could be an indicator for sensitivity to irradiation and drugs. Apoptotic propensity, therefore, is of considerable importance. While some cells exhibit a high apoptotic propensity within hours after irradiation or drug treatment (Mirkovic *et al* 1994, Weil *et al* 1996), others express significant levels of apoptosis only after days (Guo *et al* 1999). Some cells show little, if any, apoptosis and enter other death pathways. High apoptotic propensity also may reflect high sensitivity to cytotoxic treatment, but a low apoptotic propensity does not necessarily imply high resistance to cytotoxins (Olive *et al* 1996). In most tumour cells, mitotic cell death is at least as important as apoptosis, and in some cases it is the only mode of cell death.
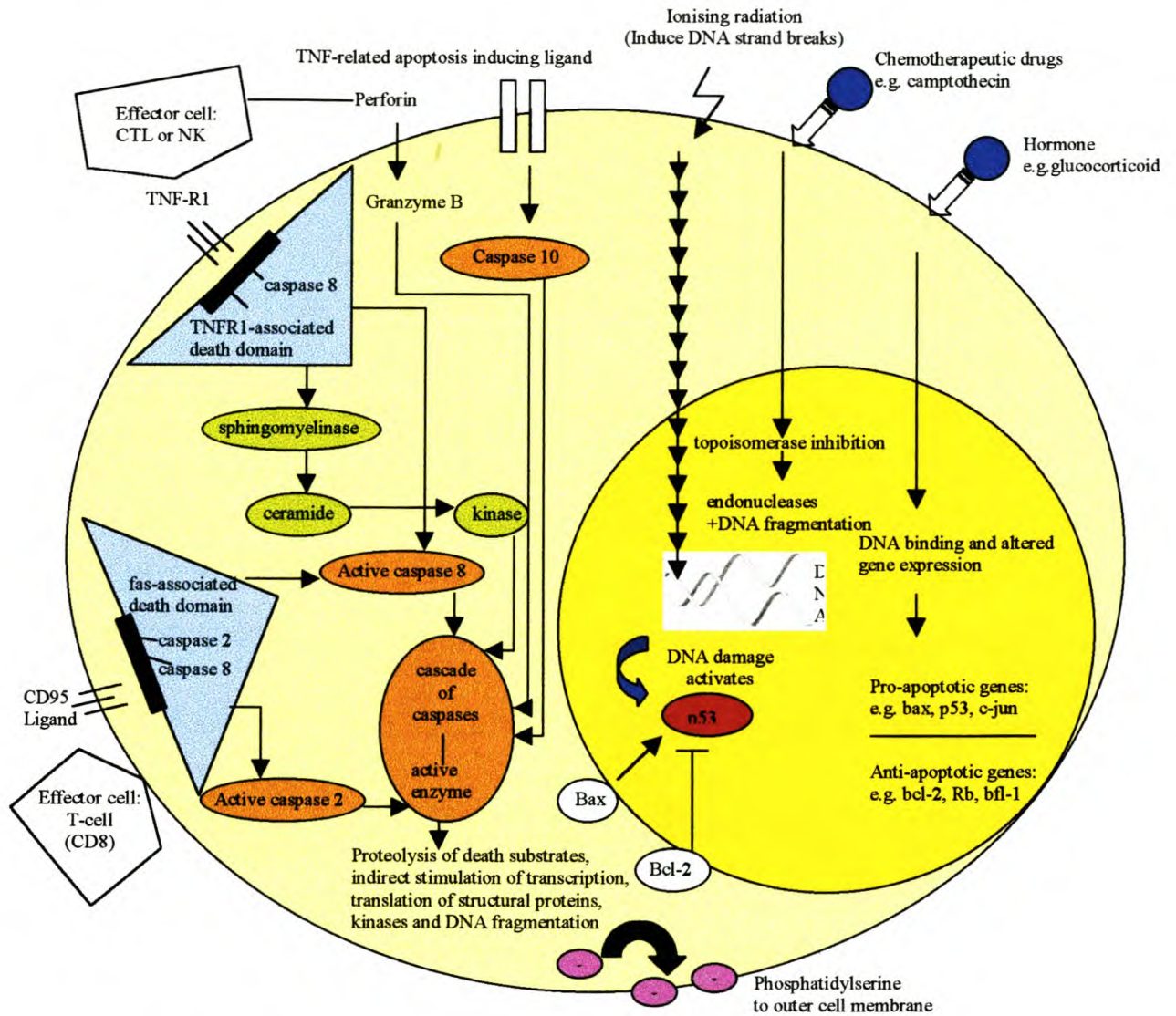
Figure 2: Basic apoptotic pathways. There are two main routes to cell death, one involving stimulation of death receptors by external ligands, and one arising within the cell and involving the mitochondria. The death receptor pathway is activated when members of the tumour necrosis factor (TNF) family of ligands bind to the receptors. This recruits adapter proteins that activate initiator caspases (eg. caspase 8), which in turn activate effector caspases, such as caspase 3. The mitochondrial pathway is activated by diverse signals, one being DNA damage. DNA damage which cannot be repaired, induces p53 protein which activates a sub-pathway that results in release of cytochrome C from the mitochondria, with subsequent involvement of the apoptosome and activation of the initiator, caspase 9. Both these pathways converge on the effector, caspase 3, to initiate proteolysis and activation of nucleases to effect cell kill. The survival factor sub-pathway normally holds apoptosis at bay by inhibiting the mitochondrial pathway through activation of members of the anti-apoptotic bcl-2 family.

DNA Repair

Ionising radiation induces various types of DNA damage, including double-strand breaks, single-strand breaks, base damage and DNA-protein crosslinks (Arrand and Michael 1992). DNA double-strand break induction and repair, and the fidelity of repair, seem to be critical factors which determine radiosensitivity in human cell lines (Nunez et al 1996). Since chemotherapeutic agents such as alkylating agents, inhibitors of DNA topoisomerase II, and inhibitors of topoisomerase I and antimetabolites are known to induce double-strand breaks, issues relating to double-strand break repair is important for the clinical oncologist.

Human cells repair DNA double-strand breaks by either of two repair pathways: the non-homologous end-joining (NHEJ) pathway, and the homologous recombination (HR) pathway (Figure 3) (Karran 2000, Thompson and Schild 2001). Homologous recombination repairs double-strand breaks using the sister chromatid as a template, removing damage in an error-free process (Liefshitz et al 1998). By contrast, non-homologous end-joining does not require homology to couple the DNA fragments in double-strand breaks. Because NHEJ simply attaches ends together and does not use a template, it is more error prone and more likely to result in chromosomal damage. Because HR requires a template, this mechanism is most efficient in the late S and G_2 phases of the cell cycle, when a sister chromatid is available as a template. Acting in coordination with the S and G_2 checkpoint controls, HR helps to eliminate chromosomal damage before cell division occurs. By contrast, NHEJ is more likely to be the repair mechanism used during G_1 (or G_0) and on unreplicated DNA in S phase. Accumulating evidence (Takata et al 1998, Essers et al 2000), indicates that the relative contribution of HR and NHEJ varies, depending on the cell lines used and the phase of the cell cycle, and that HR is more important in mammalian cells than previously thought (Sonoda et al 1999, Moynahan et al 1999).

Enzymes which are activated by DNA strand-breaks, eg. poly-ADP-ribose-polymerase (PARP) and mdm2 have been implicated in the sensing of DNA damage (Momand and Zambetti 1997). PARP is thought to indirectly activate p53, p21 and DNA-PK (Le Ruhn et al 1998).

A variety of assays exist for determining the level of DNA and chromosomal damage, and the subsequent repair. The unifying feature of these techniques is that they quantify damaged and undamaged DNA. This is then used as an indirect signal for predicting survival. A cell's ability to retain reproductive integrity is not only related to the level of damage inflicted, but is also an expression of damage repair (Roos *et al* 2000). The major limitation of repair assays is the distribution of cells in cell cycle phases. Variations in cell distribution can produce significant variations in expression of damage and, therefore, render the prediction of survival unreliable in relation to the amount of residual damage (Wlodek and Hittelman 1988). Several investigators (Andrews 1994, Perego *et al* 1998, Wang *et al* 2001) have shown that resistance to chemotherapy agents is often mediated by enhanced DNA repair, enhanced drug metabolism, or reduced drug accumulation, mechanisms that all precede the "apoptotic decision point".
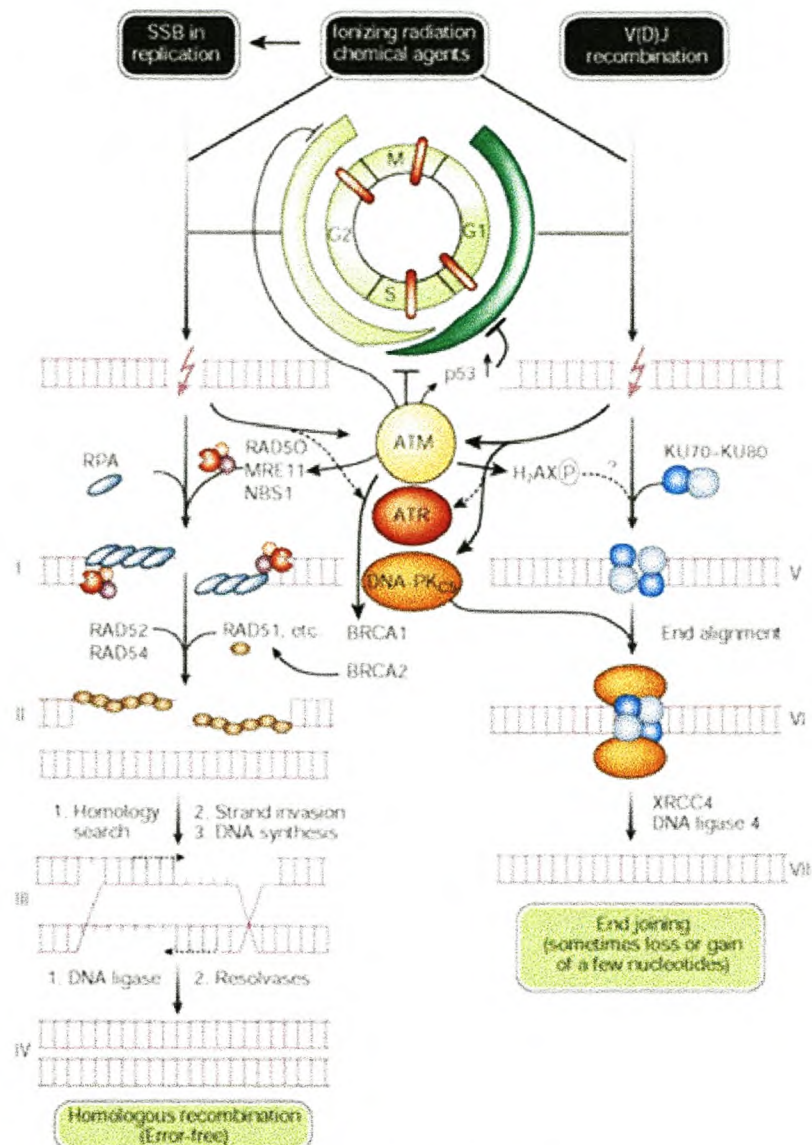
Figure 3: Schematic diagram of two important DNA repair pathways. In the non-homologous end-joining (NHEJ) pathway, the termini of a DNA double-strand break induced by ionising radiation are bound by the Ku70/80 / DNA-dependent protein kinase catalytic sub-unit complex, or by Rad52. Rejoining of the DNA ends, by filling in new nucleotides in the absence of any homology, is facilitated by DNA ligase IV and Xrcc4. DNA repair by homologous recombination (HR) occurs in G_2 after replication and involves strand invasion of the intact sister chromatid, facilitated by Rad51, using the undamaged DNA as a template.

The significance of the repair and apoptotic pathway as indicators of drug toxicity and successful cell inactivation is not clear at present. It is entirely possible that other cell death pathways are operating and that apoptotic propensity may not be strictly diagnostic of drug effectiveness. The role of repair in drug effectiveness is also obscure. It is anticipated that the present research will shed light on this question.

Thesis objective

The literature overview in the preceding introduction demonstrates that the treatment of prostate cancer remains difficult. The poor response of prostate cancer to chemotherapeutic drugs and to irradiation could be due to: inter-patient variation, inherent drug- and radioresistance, and effective DNA damage repair. The cell biological approach chosen here assesses basic damage responses, such as apoptotic propensity, checkpoint activation, p53, and repair capacity, in cell lines of defined p53 status.

The "classic" prostate cell lines, DU145, PC-3 and LNCaP, have contributed to the present understanding of prostate cancer. However, their relevance to the problem is limited because they derive from metastatic, and not from primary normal and tumour epithelium. Cell lines derived from primary benign and malignant human tumour prostate epithelium are now available (Hayward *et al* 1995, Bright *et al* 1997). These new cell lines (1532T, 1535T, 1542T, 1542N, and BPH-1) express functional characteristics of prostatic epithelial cells and provide a new opportunity to examine the role of basic cell damage responses (repair, checkpoint activation, apoptosis and associated signalling proteins, and the influence of androgen status) in cell inactivation, and their relevance in treatment. Particular importance thus is attached to the use of a more realistic cell biological model. The results from this investigation thus could provide a new basis for the understanding of irradiation and drug resistance in prostate tumours.

I have assessed the following aspects

(1) The influence of p53 status, p53 functionality and androgen dependence on cell survival in response to anticancer drugs in 1532T, 1535T, 1542T, 1542N, BPH-1 and LNCaP prostate cell lines.

(2) The role of p53 and bax/bcl-2 on apoptotic propensity induced by irradiation and anticancer drugs.

(3) The influence of DNA repair on damage response and survival.

(4) The role of apoptosis as a diagnostic parameter in drug effectiveness.

CHAPTER 2

MATERIALS AND METHODS

Cell lines

BPH-1

The epithelial cell line, BPH-1 (benign prostatic hyperplasia-1), was established from human prostate tissue obtained by transurethral resection. Primary cell cultures were immortalised with simian virus 40 (SV40) large T-antigen (Hayward *et al* 1995). BPH-1 has a cobblestone appearance in monolayer culture and grows in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), penicillin (100U/ml) and streptomycin (100µg/ml) (Highveld Biological, South Africa). The cells were obtained from Professor SW Hayward (Department of Urology, University of California).

1535-CP$_1$TX, 1532-CP$_2$TX, 1542-CP$_3$TX and 1542-NPTX

The malignant (CP$_1$, CP$_2$, CP$_3$) and benign (NP) prostate epithelial cell cultures were derived from primary adenocarcinomas of the prostate resected from 6 patients. The cells were immortalised with the E6 and E7 transforming proteins of the human papilloma virus serotype 16 (HPV-16) (Bright *et al* 1997). The growth medium consisted of WAJC 404 Medium (Gibco BRL, Scotland) containing HEPES, sodium hydrogen carbonate, zinc stabilised insulin, cholera toxin, dexamethazone, epidermal growth factor (Sigma, Germany), penicillin (100U/ml), streptomycin (100µg/ml) and 0.5% heat-inactivated foetal bovine serum (Highveld Biological), as described by Bayne and colleagues (Bayne *et al* 1998). The growth medium used by the originators of the paired prostatic cell lines, 1535N, 1535T, 1532N, 1532T, 1542N and 1542T, was keratinocyte serum-free medium (Keratinocyte SFM) containing bovine pituitary extract, epidermal growth factor, L-glutamine, HEPES buffer, antibiotics, and heat-inactivated foetal bovine serum (Bright *et al* 1997). For

economic reasons, WAJC 404 medium was used. In non-optimised conditions, the culture may select for cells which survive better under different circumstances. In addition, cell populations which grow *in vitro* for a number of generations are prone to genetic and phenotypic changes, which may be an expression of their need to adjust to a new environment. Cell growth rates, too, may differ from medium to medium. The hallmark of an adequate culture medium is that it supports high levels of cell viability. In this regard, the clonogenic assay sets the most exacting standard for the adequacy of a medium. The cells were provided by Professor JRW Masters (Prostate Research Centre, University College London) and Professor JA Macoska (Department of Urology, University of Michigan Comprehensive Cancer Center).

LNCaP.FGC

The LNCaP cell line was established from a supraclavicular lymph node metastasis of human prostatic adenocarcinoma (Horoszewicz *et al* 1983). LNCaP has a fibroblastoid morphology, low anchorage potential, is adherent but grows in aggregates and as single cells in RPMI 1640 medium supplemented with 5% heat-inactivated FBS, penicillin (100U/ml) and streptomycin (100µg/ml) (Highveld Biological). The cultures require gentle handling at all times because the cells are easily dislodged by tapping, shaking or pipetting. The low anchorage potential is also responsible for the 10-20% cell loss during media changes in long-term experiments (Horoszewicz *et al*, 1983). The cells were obtained from Professor Helmut Klocker (Department of Urology, University of Innsbruck).

RT4

The RT4 cell line, derived from a transitional-cell bladder carcinoma (Chresta *et al*, 1996), is adherent and grows as a monolayer in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100U/ml), streptomycin (100µg/ml), and 2mM glutamine (Highveld Biological). The cells were obtained from Professor JRW Masters (Prostate Research Centre, University College London).

Cell culture maintenance

Cells were maintained at 37°C and 5% CO_2 in Forma Scientific incubators and all procedures were carried out in laminar flow hoods using aseptic techniques. In all cases, LNCaP cells, once seeded, were left for at least two days, as described in Horoszewicz *et al* (1983), to attach to the surface of the cell culture flasks before experiments were conducted.

Cytotoxic Drugs

Vinblastine Through its binding to tubulin, this vinca alkaloid, $C_{46}H_{58}N_4O_9$, inhibits the assembly process of microtubules leading to dissolution of the mitotic spindle. Vinblastine (VINBLASTINE PCH, Pharmachemie Ltd) is a cell cycle-specific agent and blocks mitosis with metaphase arrest. Each vial contains 1mg/ml of Vinblastine sulphate. Vinblastine has a molecular weight of 811.00

Estramustine A stable conjugate of estradiol and nor-nitrogen mustard, estramustine (ESTRACYT 3, Pharmacia and Upjohn), in addition to inhibiting assembly of the nuclear matrix, depolymerizes microtubules by binding to tubulin- and microtubule-associated proteins, and inhibits the multidrug resistance transporter, P-glycoprotein. Estramustine, $C_{23}H_{31}CL_2NO_3$, has a molecular weight of 440.41 and is prepared as a sterile stock solution of 10mg/ml in dimethyl sulphoxide (DMSO).

Etoposide The predominant macromolecular effect of this topoisomerase II inhibitor, $C_{29}H_{32}O_{13}$, (EPOSIN IV, Pharmachemie (Pty) Ltd.) appears to be the induction of DNA strand-breaks by an interaction with DNA topoisomerase II. Etoposide was supplied as a stable solution (20mg/ml concentrate for infusion) and remained stable when added to aqueous media. Etoposide has a molecular weight of 588.58

Estramustine and Etoposide were stored at room temperature (22°C), Vinblastine at 4°C. Fresh stock solutions were used for each experiment. The criterion for drug selection was clinical potential and suitability for use in cell culture without microsomal activators.

Mycoplasma testing

Cell cultures were tested for mycoplasma contamination. Single-prostate-cell suspensions ($2x10^4$ cells) were seeded in 35mm plastic petri dishes (Corning, New York) containing 22mm glass coverslips (Chance Propper, England) to a final medium volume of 2ml, and allowed to grow for 3-5 days. The medium was then removed and the cells fixed in methanol:glacial acetic acid (3:1, v/v) for 5 minutes. The coverslips were air-dried and submerged for 1 minute in Hoechst 33258 stain (Sigma, Germany) at a concentration of 0.5µg/ml in Hanks balanced salt solution. The coverslips were then washed three times in phosphate buffered saline (PBS) before being mounted, using citric acid-disodium phosphate buffer (pH5.5), on glass microscope slides and observed under the fluorescent microscope. Mycoplasma contamination can be identified by the presence of speckled perinuclear fluorescence (Chen *et al* 1977). All cell lines used were found to be free of mycoplasma contamination.

Growth curves and cell doubling times

Exponentially growing cells were trypsinised, counted and seeded at 5×10^4 cells/ml into 25cm^2 flasks. Flasks were set up in triplicate per time point and cell numbers determined over a period of 120 hours. For counting, the cells were harvested by trypsinisation, resuspended in medium, and a known dilution in Isoton II counted with a Coulter Cell Counter (Coulter Electronics, USA). The means of the triplicate counts were plotted against time on a log scale.

Doubling time (DT) was calculated as follows:

$$DT = \frac{\ln 2}{b} = \frac{0.693}{b}$$

where b = the slope of the linear regression between cell number, on a log scale, and time, on a linear scale.

Irradiation procedure

The ^{60}Co γ-irradiation source at Tygerberg Hospital was used. Dosimetry was by Thermo Luminescent Dosimetry (TLD-chips). The beam configuration was vertical with a source-to-sample distance (SSD) of 80cm measured to the base of the experimental flasks. The field size was 30 x 30cm^2. Build-up consisted of 10ml of medium in the 25cm^2 culture flasks and a 5mm perspex sheet positioned on top of the culture flasks. The backscatter was absorbed by a 5cm thick perspex sheet.

For the clonogenic assay cell cultures were irradiated at room temperature (22°C) over a dose range of 0-10Gy at a dose rate of 1.0Gy/min. For the DNA repair experiments, the prepared gel plugs (BioRad, USA) containing cells were irradiated on ice over a dose range of 0-100Gy at a dose rate of approximately 2.92Gy/min. The SSD for this set-up was approximately 6cm and the field size was 30 x 35cm^2.

Flow cytometric determination of G_2 block expression

To establish the time of maximum G_2/M phase arrest, exponentially growing cells in 25cm^2 tissue culture flasks were exposed to a single ^{60}Co γ-irradiation dose of 7Gy. This was followed by harvesting cells at 2-hourly time intervals for up to 40 hours by trypsinisation, centrifugation and fixation in 70% ethanol at –20°C. The DNA content of the cells was measured to determine the time of maximum G_2 block expression (Ormerod 1999). Briefly, cells were stained in PBS containing 10μg/ml propidium iodide (PI) solution (Sigma, Germany) and 100μg/ml RNase (Sigma, Germany) at 37°C for 30 minutes. Analysis was done on a FACScan (Becton Dickinson, USA) flow cytometer at 488nm. Red fluorescence (PI) was collected as a linear signal and recorded as a measure of the total DNA content. Cell doublets were gated out by processing red fluorescence into area and width. Estimates of cells in the different cell cycle stages, obtained by placing markers on DNA histograms, revealed the time of maximum G_2 block expression.

Clonogenic survival assay

Near-confluent stock cultures were washed with sterile PBS, trypsinised and counted using a haemocytometer. Cells were seeded in triplicate per experiment in 25cm^2 culture flasks at numbers to yield 100-200 colonies per flask and allowed to settle for a minimum of 6 hours. Irradiation response curves were obtained by exposing cells to graded doses ranging from 0-10Gy. After an appropriate time of incubation (10-12 days) the colonies were fixed, stained and scored. Cell survival curves were fitted to the linear-quadratic survival equation:

$$Survival(S) = e^{-(\alpha D + \beta D^2)}$$

where S is the survival ratio, α and β are inactivation constants, and D is the dose in Gy. Mean surviving fractions from three independent experiments were plotted on a linear-linear scale, and the mean inactivation dose, $\overline{D}$, was used as the measure of radioresistance (Fertil $et\ al$ 1984).

$\overline{D}$, was obtained by the numerical integration of the linear quadratic function, as follows:

$$\overline{D} = \int_{o}^{\infty} S(D)dB$$

where S(D) is the dose dependence of the surviving fraction or the probability that a dose larger than D is necessary to inactivate a cell which has been randomly selected from the population (Fertil $et\ al$ 1984). $\overline{D}$ is equal to the area under the survival curve. Within the concept of $\overline{D}$, the survival curve is treated as a probability distribution of dose (Fertil $et\ al$ 1984).

$\overline{D}$ has been shown to be suitable for modelling mammalian cell survival curves (Deschavanne and Fertil 1996). The use of $\overline{D}$ is advantageous for several reasons: $\overline{D}$ is model-free and, as the radiosensitivity is evaluated by a single parameter, its statistical handling is simplified. All the experimental data are used with the same statistical weight and $\overline{D}$ minimises experimental fluctuations.

Proliferation arrest of prostatic cells by 5α-DHT

10000 cells were seeded in 24-well multiwell plates with medium containing charcoal-stripped (cs) FBS. The medium for the 1532-CP$_2$TX, 1535-CP$_1$TX, 1542-CP$_3$TX and 1542-NPTX cell lines was supplemented with 0.5% csFBS, the BPH-1 cell line with 10% csFBS, and 5% csFBS for the LNCaP cell line, which served as the positive control. 5α-dihydrotestosterone (DHT) was added a day later (2 days later in the case of LNCaP) in concentrations ranging from 0.001 – 10nM, for a period of 4 days. The experiment was stopped after 4 days and the cell growth determined by crystal violet assay, as described by Baker and colleagues (1986). Two control flasks were set up, one with charcoal-stripped FBS and one with "normal" FBS. The optical density (OD) readings, expressed as a percentage of the control OD, were plotted against concentrations of DHT.

Drug Toxicity

Drug concentrations for the various cell lines were determined as follows. Exponentially growing cells were trypsinised, counted and 10000 cells/ml seeded into 24-well multiwell plates. The cells were allowed to settle for 6 hours before the addition of varying concentrations of drug. Working solutions of the various drugs were freshly prepared for each experiment. After 24 hours of drug exposure, the medium was changed and the multiwell plates re-incubated for a further 4 days. The cells were then fixed with a solution of buffered formalin (pH 7.2) and stained with 0.01% crystal violet. The crystal violet stain was dissolved in 1ml of a 10% sodium dodecyl sulphate (SDS) solution overnight and the optical density of the extracted stain read on a Perkin-Elmer Lambda 5 spectrophotometer at 590nm. Cell survival at each drug concentration was expressed as a percentage of the control survival rate, ie. where no drug was present in the growth medium, corrected for cell plating efficiency.

Immunochemical detection of p21^{WAF1}

p21^{WAF1} detection was performed according to a modification of the protocol of Deptala and colleagues (Deptala *et al* 1999). Cells were fixed in 1% methanol-free

Immunochemical detection of p21^{WAF1}

p21^{WAF1} detection was performed according to a modification of the protocol of Deptala and colleagues (Deptala *et al* 1999). Cells were fixed in 1% methanol-free formaldehyde in PBS at 4°C for 15 minutes, washed with PBS, and permeabilised with 80% ethanol at -20°C. After fixation and re-swelling in PBS at room temperature (22°C) cells were lysed in 0.25% Triton X-100 on ice for 5 minutes. Cells were then washed with PBS containing 1% bovine serum albumin (BSA) and incubated overnight at 4°C with 100µl of 1% BSA/PBS containing 1µg of anti-p21^{WAF1} antibody (clone 2G12; PharMingen, USA), or 3µl of mouse anti-IgG1 (Dako, Denmark) in a 1:30 titre as a negative isotype control. The cells were then washed in 1% BSA/PBS and incubated in 100µl of 1% BSA/PBS containing 3µg of mouse anti-IgG1 secondary antibody (Sigma, Germany) for 1 hour in the dark at room temperature. Induction of p21^{WAF1} was visualised in dot plots of red fluorescence (PI) against green fluorescence (FITC) by flow cytometry. The p21-labelled cells were identified by their green fluorescence and were above the G$_1$-phase population. The G$_1$- and the G$_2$-phase populations were clearly identified by their lack of p21 antibody reactivity. Cell Quest software was used for the analysis. The p53 wild-type cell line, LNCaP, served as the positive control.

DNA repair (CFGE) assay

The amount of DNA double-strand break damage was determined by constant-field gel electrophoresis (CFGE), as previously described (Wlodek *et al* 1991). Confluent cultures were used to avoid S-phase variations between cell lines (Dikomey *et al* 1998). Cells were encapsulated in agarose during irradiation and repair. This procedure was optimised according to Kysela and colleagues (1993) in order to minimise non-specific DNA damage. Briefly, 1532T, 1535T, 1542T, 1542N, BPH-1 and LNCaP cells were harvested by trypsinisation and re-suspended in a 0.5% low melting point agarose solution. Aliquots of 30µl, containing ~1 x 10^5 cells were placed into each well of a disposable plug mold (BioRad, USA), and allowed to solidify at 4°C for 45 minutes. Plugs were irradiated in ice-cold DMEM containing 2% HEPES and 7.5% (w/v) NaHCO$_3$, over a dose range of 0-100Gy ^{60}Co γ-

irradiation, on ice. Samples for the determination of initial damage were immediately submitted to lysing and washing steps. The residual damage was determined by incubating the plugs at 37°C in growth medium for periods of 2 hours and 20 hours prior to lysing and washing.

For both protocols (initial and residual damage), plugs were submersed in an ice-cold lysing solution containing 50mM ethylene diamine tetra-acetic acid (EDTA), 1% sodium dodecyl sulphate and 1mg/ml proteinase K. Incubation for 1 hour at 4°C was followed by lysing at 37°C for 20 hours. Agarose plugs were then washed five times and stored in 2ml of 50mM EDTA solution.

The washed agarose plugs were then loaded onto a 20 x 20cm 0.6% agarose gel and run in 0.5 x TBE buffer for 24 hours at a constant field strength of 1.2V/cm. Gels were stained with ethidium bromide (0.5µg/ml in 0.5 x TBE) and subjected to fluorometric analysis with a GeneSnap (VacuTec) image analysis system. Three independent experiments were performed for each cell line. The fraction of DNA released from the plug was obtained from the following equation: $F_{rel} = fl_{rel} / (fl_{plug} + fl_{rel})$, where fl_{rel} and fl_{plug} correspond to fluorescence measured in the lane (DNA released) and in the plug, respectively. Untreated control samples were used for each sample sub-set to subtract background fluorescence caused by non-specific DNA degradation.

Dose-response curves were obtained by plotting dose (Gy) vs. the fraction of DNA released (F_{rel}) as calculated above, representing initial damage (0 hours), residual damage (2 hours) and residual damage (20 hours). Since data could not be fitted by linear regression, data points were connected and the area under the curve (AUC) was calculated for each curve using GraphPad Prism (GraphPad software, USA). DNA double-strand break (dsb) rejoining capacity after 2 hours or 20 hours incubation was then determined as the ratio AUC_0/AUC_2 or AUC_0/AUC_{20}, where AUC_0 is the area under the curve at 0 hours.

Measurement of *in vitro* apoptosis

The apoptosis assay was performed, as described previously (Akudugu and Bohm 2000). Single-prostate-cell suspensions ($2x10^4$ cells) were plated into 35mm plastic

petri dishes (Corning, New York) containing 22mm glass coverslips (Chance Propper, England) to a final medium volume of 2ml. The cells were rendered apoptotic by exposure to TD_{50} concentrations of Etoposide, Vinblastine and Estramustine. Treated and untreated cultures were fixed in methanol:acetic acid (3:1, v/v) at intervals between 0 and 48 hours, air-dried, stained with acridine orange, and the coverslips mounted on glass microscope slides for fluorescent microscopy. Adherent cells were harvested and combined with cells floating in the growth medium. A preliminary set of experiments was performed to establish the time at which each cell line, exposed to a TD_{50} concentration of drug, had expressed maximum apoptosis.

A minimum of 500 cells was evaluated per sample for each time point and the frequency of apoptotic cells, corrected for spontaneous apoptosis in untreated samples, was scored according to criteria described elsewhere (Mirkovic *et al* 1994, Weil *et al* 1996). These included cells with either pyknotic, fragmented or crescent-shaped nuclei, overall shrinkage, or membrane-bound bodies containing pyknotic nuclei that were smaller than the normal surrounding nuclei. Three independent experiments were subsequently performed for each cell line and drug, and the frequency of apoptotic events expressed as the mean (±SD) ratio of apoptotic cells to the total number of cells scored.

Acridine orange staining to identify morphological features of apoptosis

The DNA dye, acridine orange (Sigma, Germany), was used to identify the characteristic chromatin changes associated with apoptosis. Acridine orange is retained in cells that have an intact cell membrane, which includes the majority of apoptotic cells, but excludes necrotic cells or very late stage apoptotic cells (Figure 4).
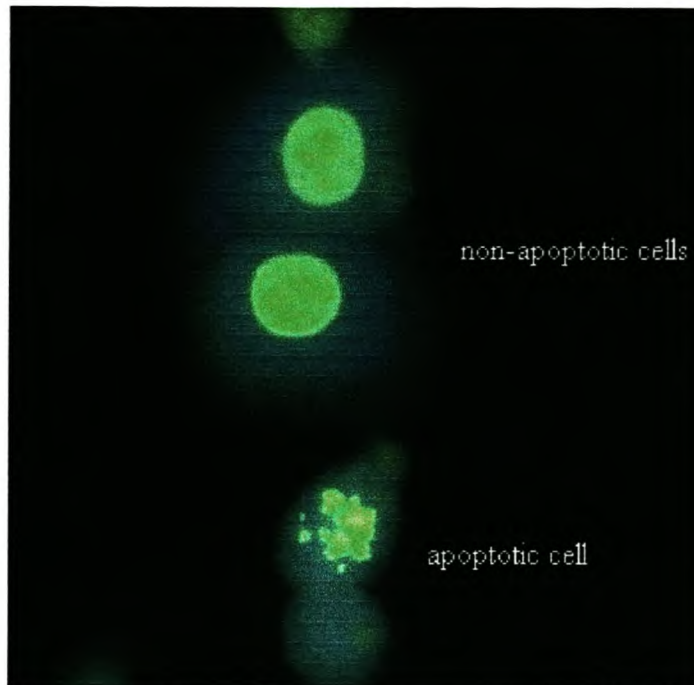
Figure 4: Apoptotic and non-apoptotic cells of the 1542T prostate line, following exposure to a TD_{50} concentration of the apoptosis-inducing drug, etoposide.

Bcl-2 and Bax analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine changes in the expression of bax and bcl-2 proteins. This method separates proteins by the relative distance they migrate through a polyacrylamide gel matrix, based on their molecular weights.

Sample preparation

Apoptotic cells detached from the monolayer after drug treatment were collected by centrifugation and combined with the monolayer cells that had been detached by gentle scraping. The cells (2×10^6) were washed in ice-cold PBS, resuspended in 200µl of PBS containing protease inhibitors, phenylmethylsulfonylfluoride (10µl/ml; Sigma, Germany) and aprotinin (30µl/ml; Sigma, Germany), and sonicated (Branson, USA) for 10 seconds at an amplitude of 27µm. Protein concentration was determined by Bradford dye binding assay, and 25µg of protein was used per sample.

Preparation of acrylamide gels

A Hoefer SE200 (Hoefer Scientific Instruments, USA) mighty small vertical slab gel electrophoresis unit was used. A 12% separating gel, with a 4.5% stacking gel, was used for optimum resolution of the bax and bcl-2 proteins. Briefly, the separating gel was prepared by mixing appropriate volumes of the following solutions: 4ml of bisacrylamide (37.5 : 1% w/v), 2.5ml of Tris 1.5M (pH 8.8), 3.35ml of distilled water, and 0.1ml of 10% SDS. After degassing the mixture, 50µl of freshly prepared 10% ammonium persulphate and 10µl of TEMED were added to initiate the reaction. The stacking gel was prepared as follows: 975µl of bisacrylamide (37.5 : 1% w/v), 625µl of Tris 1.0M (pH 6.8), 50µl of 10% SDS, and 3.375ml of distilled water. After degassing the mixture, 25µl of freshly prepared 10% ammonium persulphate and 10µl of TEMED were added to initiate the reaction. The tank buffer was prepared as follows: 3.03gm Trizma base, 14.4gm glycine, and 10ml of 10% SDS were dissolved in 1 litre of distilled water. The final pH was 8.4 - 8.6

Samples were boiled for 5 minutes in SDS sample buffer before loading onto the gel. In addition, 10µl of a precision pre-stained molecular weight marker (10-250kD; BioRad, USA) was also loaded. Gels were run for approximately 50 minutes at 200V until the dye-front reached the end of the gel.

Western transfer and immunoblot

Proteins were transferred onto a polyvinylidine difluoride (PVDF) membrane (BioRad, USA). This microporous membrane has a high protein binding capacity without degradation of the protein during elution and also has a high mechanical strength. The membrane was pre-soaked in methanol for 30 seconds and then rinsed in transfer buffer to remove the methanol (the PVDF membrane is extremely hydrophobic and will not wet in aqueous solutions unless pre-wet with methanol). The proteins were transferred from the gel to the membrane by electroblotting in transfer buffer containing: glycine (192mM), Tris-HCl (10mM) and methanol (20%), at 200mA for 1½ hours at room temperature. Ponceau Red (Sigma, Germany) staining of the PVDF membrane confirmed even loading of samples. The membrane was then blocked in Tris buffered saline-Tween 20 (TBST) containing 10% non-fat

milk powder, for 2 hours at room temperature. Five washes in Tris buffered saline (TBS)/Tween 20 solution followed, before the primary antibody was added and the membrane left rotating overnight at 4°C. Incubation in the primary antibodies [mouse anti-human bcl-2 (Ab-124) monoclonal antibody (DAKO, Denmark) and mouse IgG_{2B} bax (B-9) monoclonal antibody (Santa Cruz Biotechnology, Germany)] was followed by several washes in TBS/Tween 20 solution, before the addition of the anti-mouse IgG horse radish peroxidase antibody (Amersham, England) and incubation for 1 hour at room temperature. The membrane was then washed several times in TBS/Tween 20 solution, before protein detection.

The ECL Western blotting detection system

Proteins were detected using the enhanced chemiluminescence (ECL) detection system (Amersham). This is a rapid, highly sensitive, non-radioactive method for the detection of immobilised proteins. In the ECL reaction the substrate, luminol, is oxidised by a horse radish peroxidase-catalysed reaction resulting in blue light emission. This can be detected using blue light sensitive film.

After 60 seconds incubation with the ECL detection reagents, the membrane was removed from the liquid, cling-wrapped and exposed to Hyperfilm ECL (Amersham). Exposure times varied from 1 minute to 30 minutes.

Ponceau S staining of PVDF membranes

PVDF membranes were stained with Ponceau S (Sigma, Germany) to expose the protein bands and allow for an assessment of sample loading. Following protein transfer by electroblotting, membranes were soaked in Ponceau S for 60 seconds and rinsed in distilled water, before blocking with TBST containing 10% non-fat milk powder.

Stripping and re-probing Western blots

The ECL detection system allows membranes to be stripped and re-probed with a different antibody so that the same samples can be analysed for the expression of two independent proteins.

The membranes were submerged in stripping buffer containing 100mM β-mercaptoethanol, 2% (w/v) SDS and 62.5mM Tris-HCl (pH 6.7), and incubated, with occasional agitation, at 60°C for 30 minutes before being washed in TBST at room temperature for 20 minutes. Blocking for 1 hour at room temperature in milk block buffer followed before re-probing with primary and secondary antibodies, as described previously.

Data evaluation

Data are presented as the mean ± standard deviation of three independent experiments as indicated by error bars (Figures), and each experiment was repeated at least three times. Statistical analysis and data fitting were performed by means of GraphPad Prism (GraphPad Software, San Diego, USA). A two-sided t test was used to compare the means between sample groups.

CHAPTER 3

RESULTS

Growth characteristics

The doubling times for the malignant cell lines, 1532T, 1535T and 1542T, were found to be in the range of 28-29 hours. As expected, the normal cell line, 1542N, has a longer doubling time (36 hours), whereas the benign BPH-1 cell line has a very short doubling time (16 hours). The metastatic wild-type cell line, LNCaP, has a doubling time of 45 hours.

Cell cycle changes after irradiation:

When cells are exposed to ionising radiation, the cell cycle is blocked at the G_1/S and G_2/M transitions depending upon the p53 status of the cells.

In the 1532T, 1535T, 1542T, 1542N, and BPH-1 cells, which are p53 defective, irradiation instead generates a block at the G_2/M transition. As expected, the p53 wild-type cell line, LNCaP, showed a very pronounced delay at the G_1/S transition and a smaller G_2/M phase delay.

In the p53-defective cell lines, 1532T, 1535T, 1542T, 1542N, and BPH-1, the G_2/M delay was maximally expressed at 32, 24, 30, 36 and 14 hours, respectively, after irradiation.

In the p53 wild-type cell line, LNCaP, the G_1 and G_2 blocks maxima occurred after 40 hours.

Radiosensitivity

The cellular radiosensitivity expressed in terms of the surviving fraction at 2Gy (SF2) was determined by clonogenic assay. Cell survival data for the 6 human prostate cell

lines were fitted to the linear-quadratic model and are presented in Figure 5. The SF2 values varied from 0.28 to 0.54. The cell inactivation parameters are summarised in Table 1. The LNCaP and 1542T cell lines are the most radiosensitive, with SF2 values of 0.28 and 0.30, respectively. The 1532T and BPH-1 cell lines are the most radioresistant, with SF2 values of 0.52 and 0.54, respectively, while the 1542N and 1535T cell lines assume intermediate positions with SF2 values of 0.37 and 0.44, respectively.

Cellular radiosensitivity expressed as the mean inactivation dose, $\overline{D}$, is defined as the area under the survival curve when plotted on a linear·linear scale (Figure 6) (Fertil *et al* 1984). $\overline{D}$-values for the irradiated panel of cell lines are shown to vary from 1.62-2.77Gy (Table 1).

Table 1: Summary of cell inactivation parameters for 6 prostate cell lines determined by clonogenic assay after exposure to ^{60}Co γ-irradiation.

Cell line	α (Gy^{-1})	β (Gy^{-2})	$\overline{D}$ (Gy)	SF2
BPH-1	0.266	0.021	2.77	0.54
1532T	0.292	0.017	2.72	0.52
1535T	0.358	0.027	2.40	0.44
1542N	0.502	0.007	2.28	0.37
1542T	0.642	0.014	1.84	0.30
LNCaP	0.632	0.004	1.62	0.28
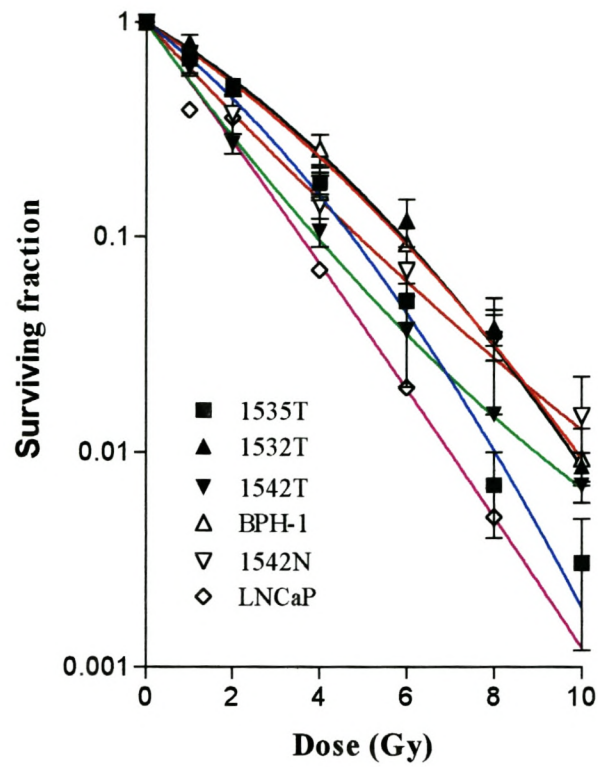
Figure 5: Clonogenic survival curves for 6 prostate cell lines after ^{60}Co γ-irradiation. Symbols represent the mean (± SD) surviving fraction from 3 separate experiments. Survival curves were obtained by fitting experimental data to the linear-quadratic model.
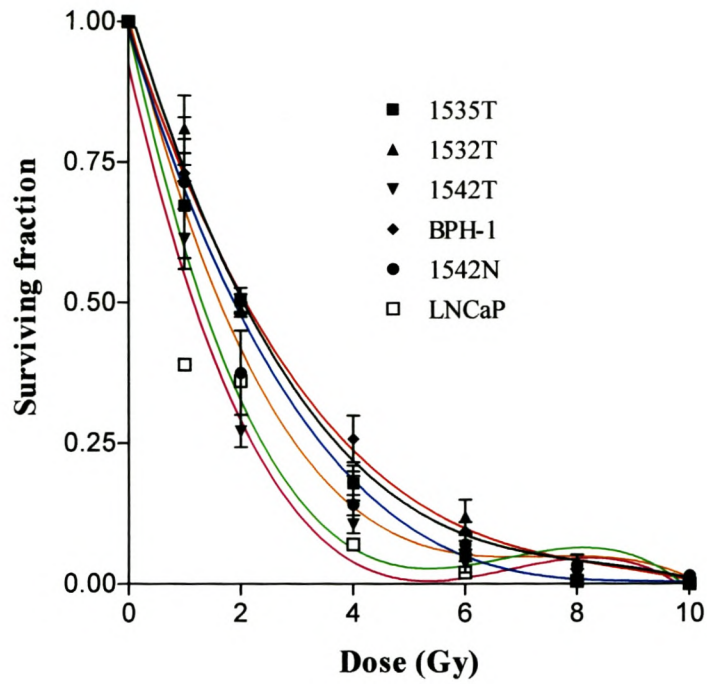
Figure 6: Survival curves plotted on a linear-linear scale for 6 prostate cell lines after ^{60}Co γ-irradiation. Symbols represent the mean (± SD) surviving fraction from 3 separate experiments. Radiosensitivity was expressed in terms of mean inactivation dose, ($\overline{\text{D}}$), which is defined as the area under the survival curve of each cell line.

Androgen sensitivity

The crystal violet vital dye staining assay shows that addition of 5α-dihydrotestosterone (DHT) has no significant effect on cell growth in the 1532T, 1535T, 1542T, 1542N and BPH-1 cell lines (Figure 7). In these cell lines growth in charcoal-stripped foetal bovine serum (csFBS) equalled or exceeded growth in the presence of 0.001-10nM DHT, indicating that they are androgen independent. However, when the LNCaP cells were subjected to charcoal-stripped medium and DHT was then added at concentrations of 0.001-1.0nM, growth was restored from 50% in csFBS to a level of 80% of control (FBS) (Figure 7). This confirms that LNCaP cells are androgen sensitive. The fact that DHT did not restore growth to 100% indicates that factors, other than steroids, removed by charcoal treatment, were essential for optimal growth.

Androgen sensitivity
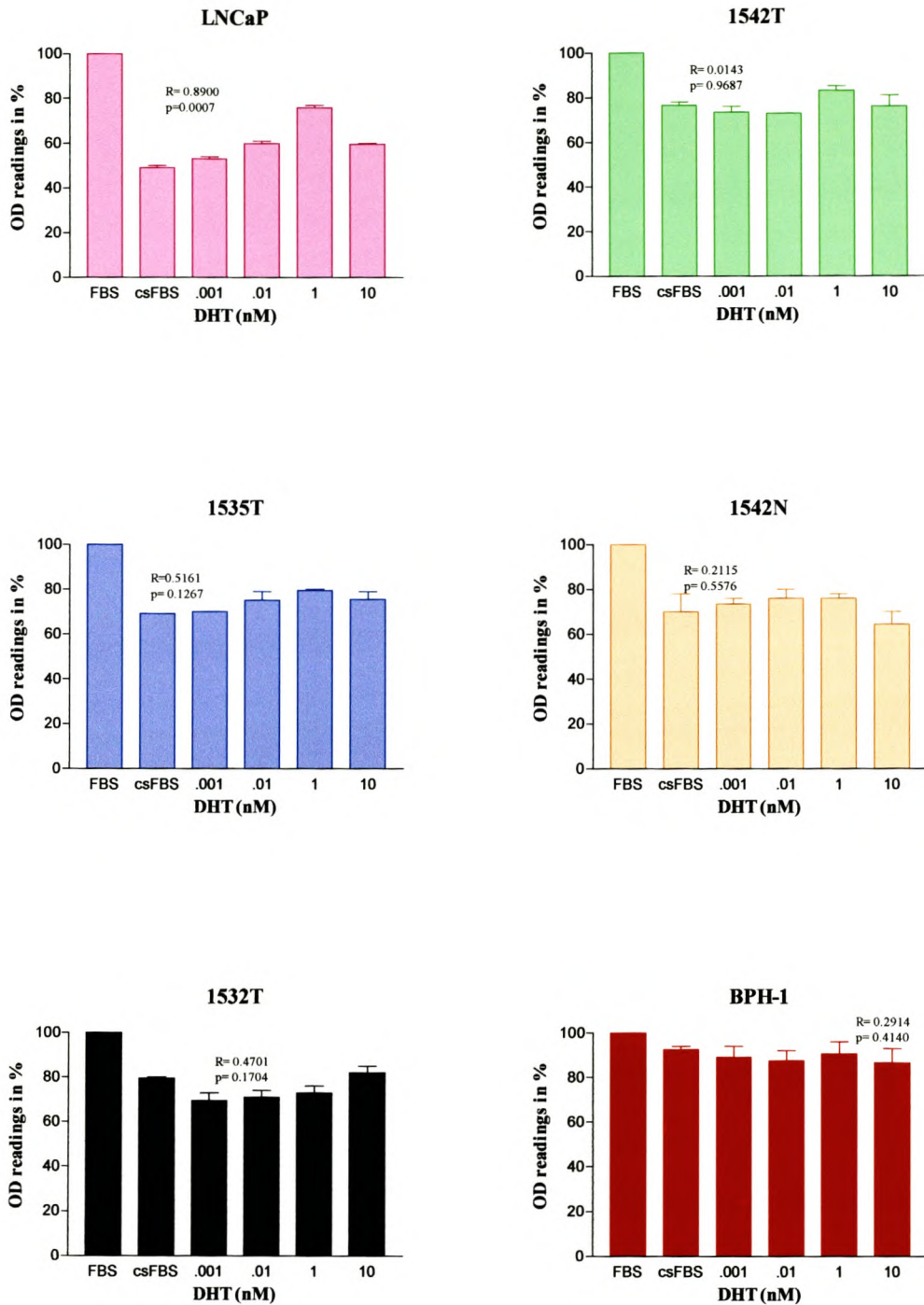
Figure 7: The effect of DHT addition (nM) to charcoal-stripped medium on the proliferation of 6 prostate cancer cell lines, measured by crystal violet dye staining assay.

Induction of p21

Induction of p21^{WAF1} gene by ^{60}Co γ-irradiation was used to assess p53 function. The p53 wild-type cell line, LNCaP, expresses functional p53 as indicated by the induction of the p21 target gene (Figure 8). No appreciable p21 induction was apparent during the initial 2.5 hours post-irradiation but p21 induction reached a peak 7.5 hours post-irradiation (7Gy ^{60}Co γ-irradiation).

No significant p21 induction was evident in the 1532T, 1535T, 1542T and 1542N cell lines immortalised with the E6 and E7 transforming genes of HPV-16, or the BPH-1 cells which have been immortalised with the large T-antigen gene of SV40. No p53 - independent induction of p21 was evident either.
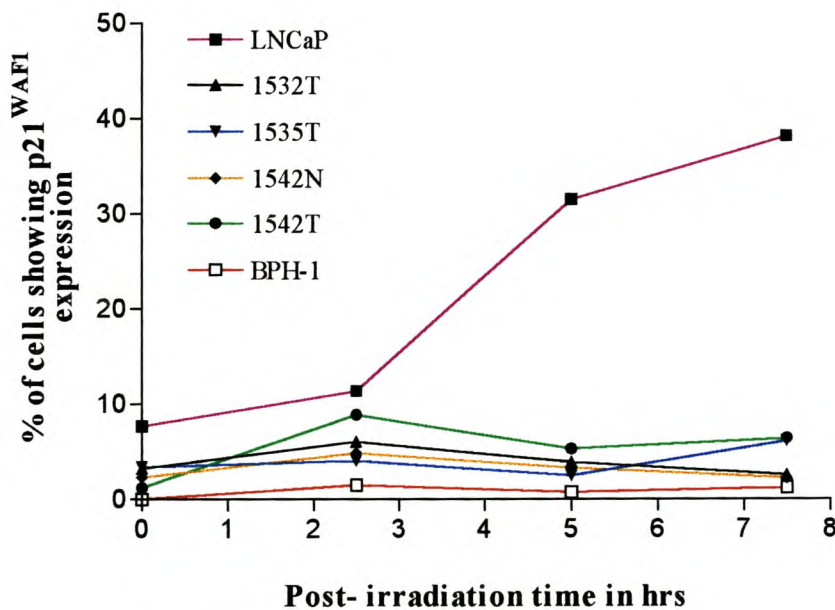
Figure 8: The response of prostate cell lines to 7Gy ^{60}Co γ-irradiation in terms of nuclear accumulation of p21^{WAF1}

Drug toxicity

Results from crystal violet assay data demonstrate that cell lines differ in drug sensitivity. Using TD_{50} data the cell lines can be ranked as follows:

Etoposide: LNCaP>BPH>1532T>1535T>1542N>1542T

Vinblastine: LNCaP>BPH>1532T>1542N>1535T>1542T

Estramustine: BPH>LNCaP>1535T>1542N>1532T>1542T

It is apparent that LNCaP is the most resistant cell line against vinblastine and etoposide, and the second most resistant against estramustine. The 1542T cell line is by far the most sensitive cell line against all 3 drugs (Figure 9, Table 2).

Vinblastine is the most toxic drug with TD_{50} values ranging from 0.15 - 3.70nM. Etoposide takes an intermediate position with TD_{50} values ranging from 0.15 - 4.70μM. Estramustine is the least toxic drug shown by TD_{50} values ranging from 1.45 - 4.30μM.

Table 2: TD_{50} drug concentrations for 6 human prostate cell lines.

Cell lines	Etoposide (μM)	Vinblastine (nM)	Estramustine (μM)
LNCaP	4.70	3.70	3.90
BPH-1	0.70	3.34	4.30
1532T	0.57	0.29	1.76
1535T	0.45	0.26	2.51
1542T	0.15	0.15	1.45
1542N	0.39	0.28	1.85
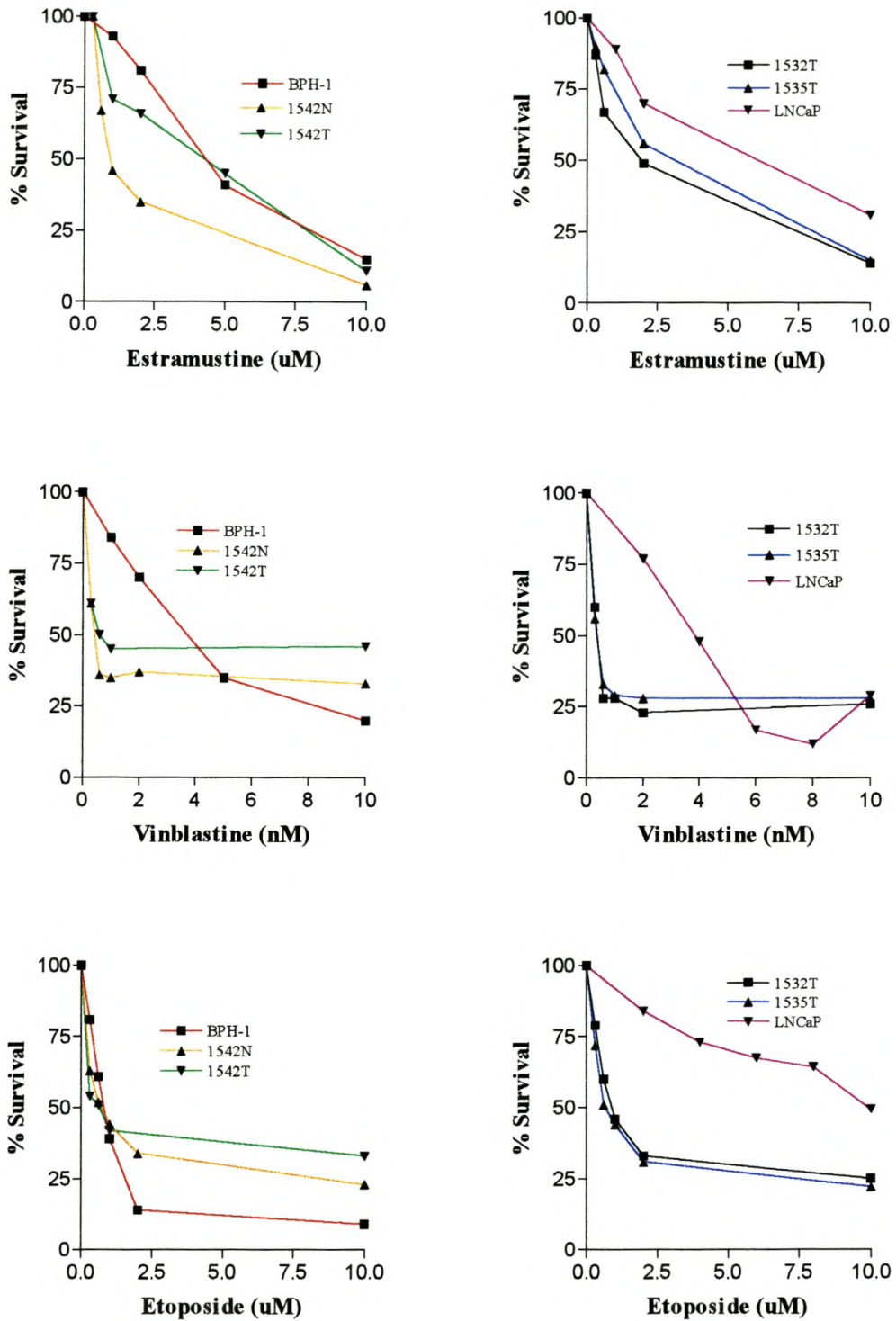
Figure 9: Dose response curves of estramustine, vinblastine and etoposide for 6 human prostate cell lines.

DNA dsb repair capacity

DNA repair was assayed by constant-field gel electrophoresis which measures the concentration of mobile (low molecular weight) DNA generated by double-strand breaks after high doses of irradiation (10-100Gy). A typical gel is shown in Figure 10. Lanes 1 to 3 show DNA after 0Gy irradiation. The effect of irradiation dose on DNA mobility after 10, 20, 40, 80 and 100Gy is shown in lanes 4 to 6, lanes 7 to 9, lanes 10 to 12, lanes 13 to 15, and lanes 16 to 18, respectively. These results were used to plot the dose-response curves.

The dose-response curves for initial (0 hour) and residual (after 2 hours and 20 hours of repair) DNA damage are presented as a plot of the fractions of DNA released (F_{rel}) against irradiation dose (Figure 11). The areas under the initial damage curves (no repair) range between 24.38 and 39.67. These results are summarised in Table 3.

To assess the rate of double-strand break repair, ratios of AUC values representing initial damage versus residual damage after 2 hours and after 20 hours were calculated. These results are summarised in Table 4. After 2 hours of repair, the six cell lines show repair ratios (AUC_0/AUC_2) ranging from 1.70 - 3.83. The 20 hours repair ratios (AUC_0/AUC_{20}) range from 3.77 - 12.02.

After 2 hours DNA repair the more radioresistant cell lines, BPH-1 and 1532T, (with SF2 values of 0.54 and 0.52, respectively) show higher levels of DNA dsb rejoining than the more radiosensitive cell lines, LNCaP and 1542T. The 2 hours repair and radiosensitivity, measured in terms of $\overline{D}$, were significantly correlated, giving a regression coefficient of r = 0.92 (p = 0.009) (Figure 12). Increases in the α coefficients would imply increased sensitivity to radiation, and low repair proficiency. No relationship was apparent between the 20 hours DNA repair capacity and radiosensitivity (Figure 13), and between initial DNA damage and radiosensitivity (Figure 14). This indicates that, in terms of clonogenic survival, radiosensitivity in these prostate cell lines is related to the fast (2 hours) DNA dsb repair component, and not to the slow (20 hours) DNA repair component. The 20 hours repair component has been found to be diagnostic of radiosensitivity in a wide variety of tumour cell lines of different origin.
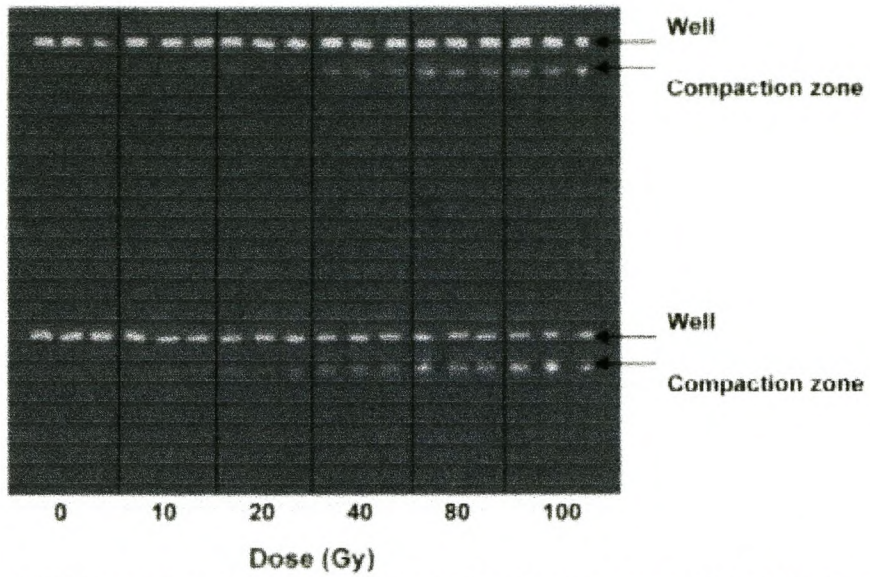
Figure 10: CFGE gel showing DNA released for the dose range 0-100Gy in a single prostate cell line.
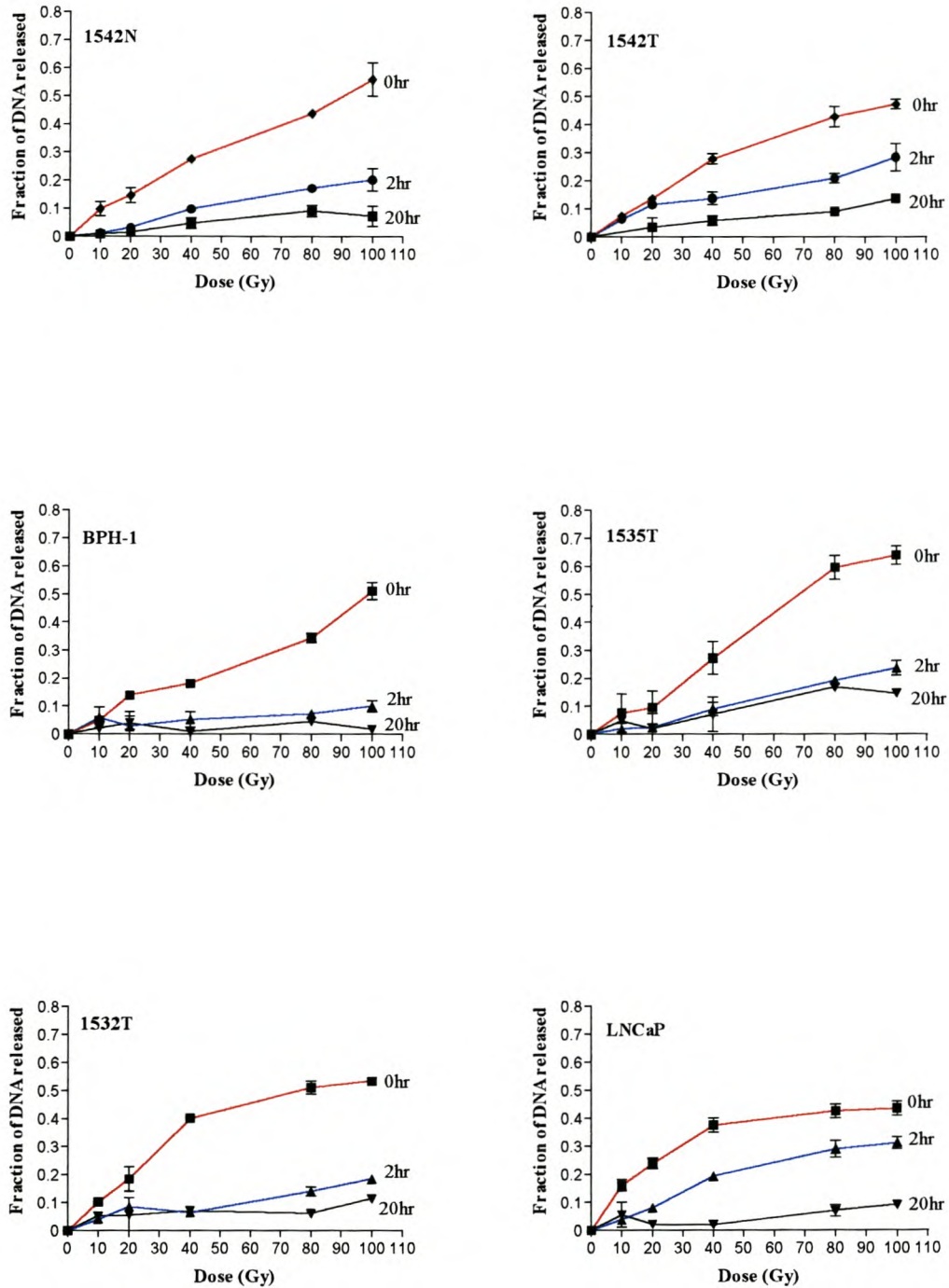
Figure 11: Dose response curves for initial (0hr) and residual (2hr and 20hr) DNA double-strand breaks in 6 prostate cell lines after 0-100Gy ^{60}Co γ-irradiation, as determined by fluorescent densitometry of ethidium bromide stained CFGE gels. Area under the curve (AUC) was calculated for each dose response curve and is given in Table 3.

Table 3: Areas under the dose-response curves (AUC) calculated from fractions of DNA released, against irradiation dose, in the range 0-100Gy for 0hr, 2hr and 20hr after irradiation (Figure 11), and radiosensitivity measured by the mean inactivation dose, $\overline{D}$, (area under survival dose response curve plotted on a linear-linear scale) for 6 human prostate cancer cell lines.

	1532T	1535T	1542N	1542T	BPH-1	LNCaP
Initial DNA damage	33.94	39.67	30.66	30.72	24.38	33.41
2hr repair	8.86	14.33	10.62	16.61	8.07	19.70
20hr repair	7.26	10.51	7.05	6.97	3.79	2.78
$\overline{D}$ (Gy)	2.72	2.40	2.28	1.84	2.77	1.62

Table 4: Ratios of initial vs residual DNA damage derived from AUC data (Table 3) for various repair times.

	1532T	1535T	1542N	1542T	BPH-1	LNCaP
2hr repair	3.83	2.77	2.89	1.85	3.02	1.70
20hr repair	4.67	3.77	4.35	4.41	6.43	12.02
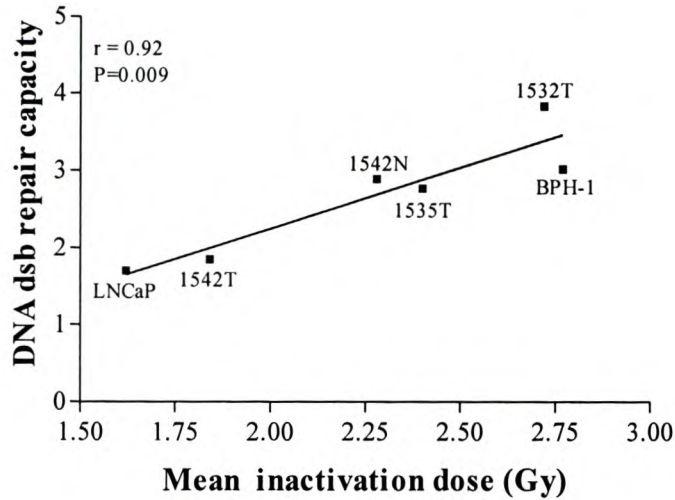
Figure 12: Plot of DNA repair capacity as a function of radiosensitivity in 6 prostate cell lines. Repair capacity is defined as the ratio of the area under the repair curve at zero repair time, to the area under the repair curve after 2 hours of repair time. Radiosensitivity is expressed as the mean inactivation dose, $\overline{D}$, which is given by the area under the cell survival dose response curve, plotted on a linear-linear scale.
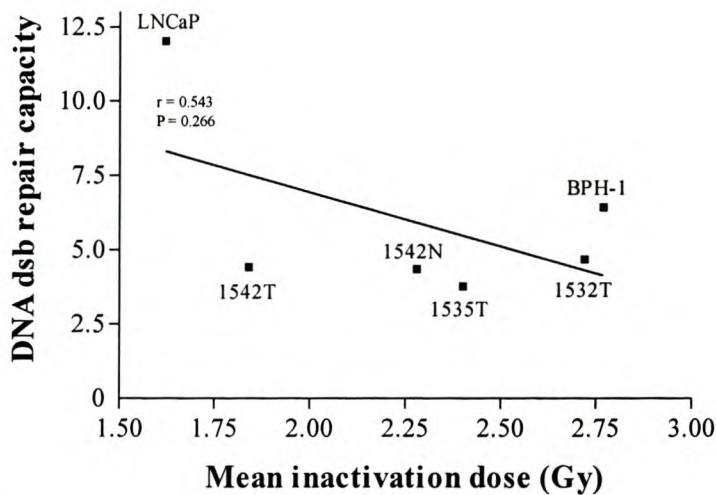
Figure 13: Plot of DNA repair capacity as a function of radiosensitivity in 6 prostate cell lines. Repair capacity is defined as the ratio of the area under the repair curve at zero repair time, to the area under the repair curve after 20 hours of repair time. Radiosensitivity is expressed as the mean inactivation dose, $\overline{D}$, which is given by the area under the cell survival dose response curve, plotted on a linear-linear scale.
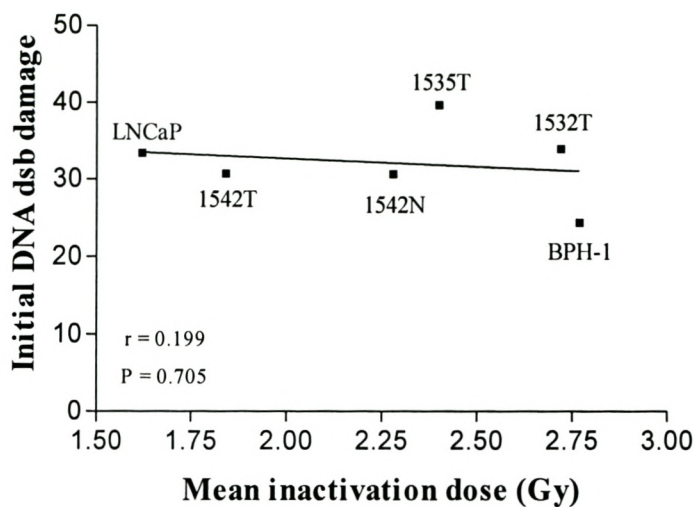
Figure 14: Plot of initial (0hr) DNA dsb damage induced by high doses of ^{60}Co γ-irradiation, as a function of radiosensitivity in 6 prostate cell lines. Initial DNA dsb damage was measured by the CFGE assay. Radiosensitivity is expressed as the mean inactivation dose, $\overline{D}$, which is given by the area under the cell survival dose response curve, plotted on a linear-linear scale.

Induction of apoptosis after drug exposure

The incidence of apoptosis in response to drug exposure, at TD_{50} concentrations, was assessed over a period of 48 hours in 6 prostate cell lines, and can be ranked as follows:

Etoposide: 1535T>1532T>1542T>1542N>LNCaP>BPH-1

Vinblastine: LNCaP>1542T>1535T>1532T>1542N>BPH-1

Estramustine: LNCaP>1542N>1542T> BPH-1>1532T>1535T

Apoptosis was found to be dose- and time-dependent in all cell lines. Apoptosis levels ranged from 0.1 - 12.1%, 3.0 - 6.0% and 0.1 - 8.5% for etoposide, estramustine, and vinblastine exposures, respectively (Table 5). The BPH-1 cell line yielded the lowest percentage of apoptotic cells after exposure to a given drug dose of vinblastine and etoposide. The LNCaP cell line yielded the highest percentage of apoptotic cells after exposure to a given drug dose of vinblastine and estramustine. Estramustine emerged as the least toxic drug for this panel of cell lines, showing low apoptosis level with an upper range of 6.0%. Vinblastine, the most toxic drug employed in this study, assumed an intermediate position with an upper apoptosis range of 8.5%, whilst etoposide showed the highest level, with an upper range of 12.1%. Maximum apoptosis levels for the prostate cell lines occurred between 6 and 48 hours after addition of the drug (Figure 15).

Table 5: Percentage maximum apoptosis, measured by fluorescent microscopy, for 6 prostate cell lines exposed to TD_{50} concentrations of etoposide, vinblastine and estramustine over a period of 0-48 hours. The time of maximum expression of apoptosis (in hours) for each cell line, versus each drug, appears in brackets.

Cell lines	Etoposide	Vinblastine	Estramustine
LNCaP	4.4 (12h)	8.5 (24h)	6.0 (36h)
BPH-1	0.1 (24h)	0.1 (48h)	4.0 (12h)
1532T	8.0 (6h)	3.3 (12h)	3.6 (6h)
1535T	12.1 (6h)	4.0 (36h)	3.0 (24h)
1542T	6.0 (24h)	6.0 (36h)	4.8 (36h)
1542N	4.8 (12h)	3.1 (24h)	5.1 (12h)
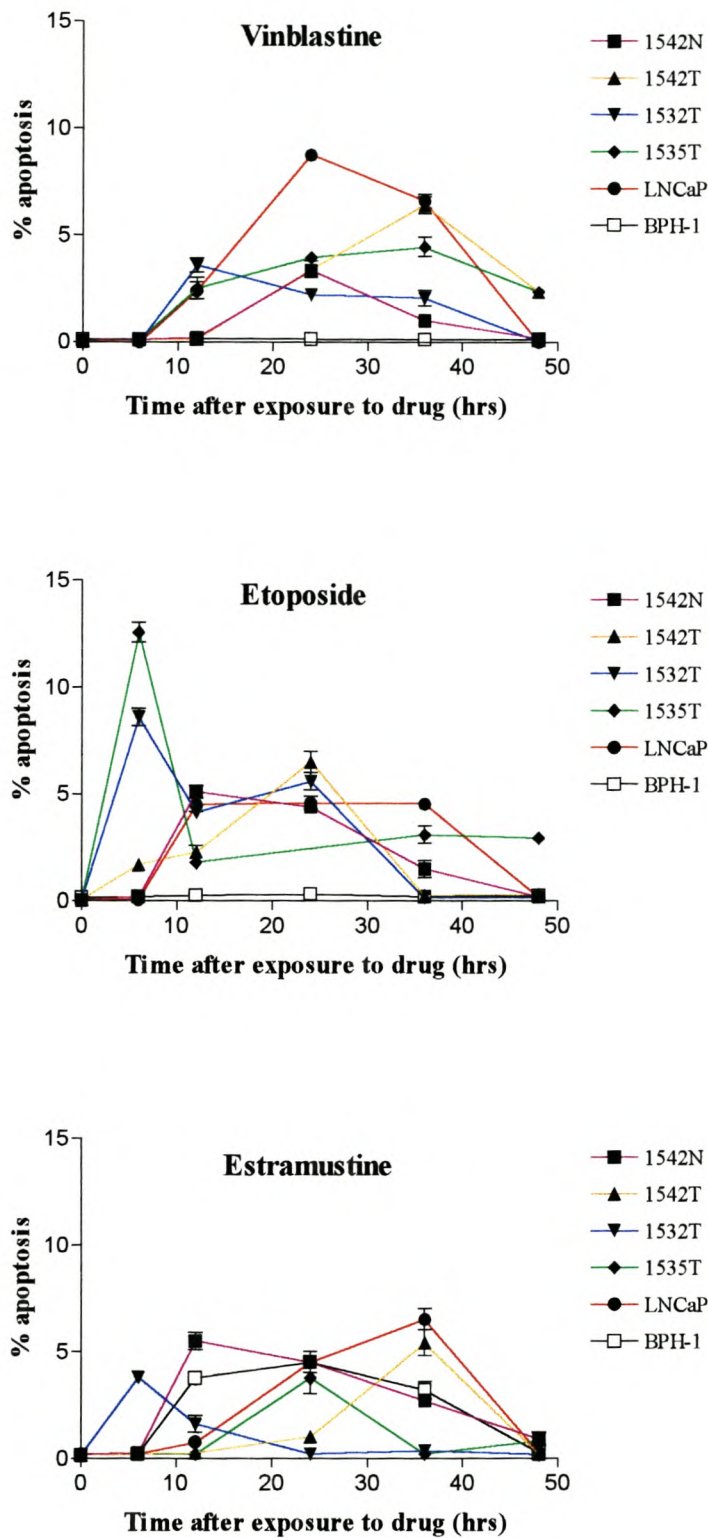
Figure 15: Percentage apoptosis induced in 6 prostate cell lines exposed to TD_{50} concentrations of etoposide, estramustine and vinblastine, over a period of 48hr. Each data point was corrected for spontaneous apoptosis in untreated cultures incubated for corresponding periods of time.

Induction of apoptosis after ^{60}Co γ-irradiation

The percent apoptosis induced by doses of gamma irradiation resulting in 50% survival at 48 hours post-irradiation can be ranked as follows:

1532T>1535T>1542T>1542N>BPH-1>LNCaP

The data for apoptosis shows a dose- and time-dependent occurrence of this phenomenon in all cell lines.

Apoptosis levels ranged from 1.3 - 7%. The LNCaP cell line yielded the lowest percentage of apoptotic cells after exposure. The 1532T cell line yielded the highest percentage of apoptotic cells after exposure. Maximum apoptosis levels for the prostate cell lines occurred at varying times between 6 and 48 hours. Figure 16 also shows that apoptosis does not rank these cells according to radiosensitivity.
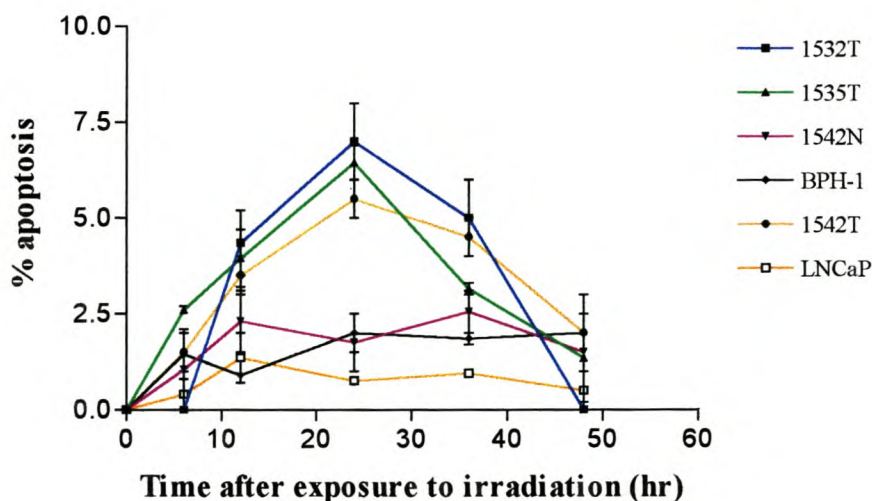
Figure 16: Percentage apoptosis induced over a period of 48hr in 6 prostate cell lines exposed to doses of ^{60}Co γ-irradiation resulting in 50% survival. Each data point was corrected for spontaneous apoptosis in untreated cultures incubated for corresponding periods of time.

Expression of Bax and Bcl-2: basal levels and the effect of DNA damage

Bax and bcl-2 expression was determined after etoposide, vinblastine and estramustine treatment at TD_{50} concentrations. Samples were taken at the time of maximum apoptosis expression and were then analysed for bax/bcl-2 expression.

Immunoblotting demonstrated that bax and bcl-2 proteins were expressed at a basal level in all the cell lines, and that the levels, analysed by densitometry (UN-SCAN-IT_{TM} gel automated digitising system, Silk Scientific, USA), were unaffected by exposure to etoposide, vinblastine and estramustine (Figures 17, 18, and 19). Exposures to TD_{50} doses of etoposide, vinblastine and estramustine at the time of maximum apoptosis did not change the bax/bcl-2 ratio. The bladder carcinoma cell line, RT4 (Chresta *et al* 1996), served as a positive control for both bcl-2 and bax detection, using our antibody, and was run on the same gel.
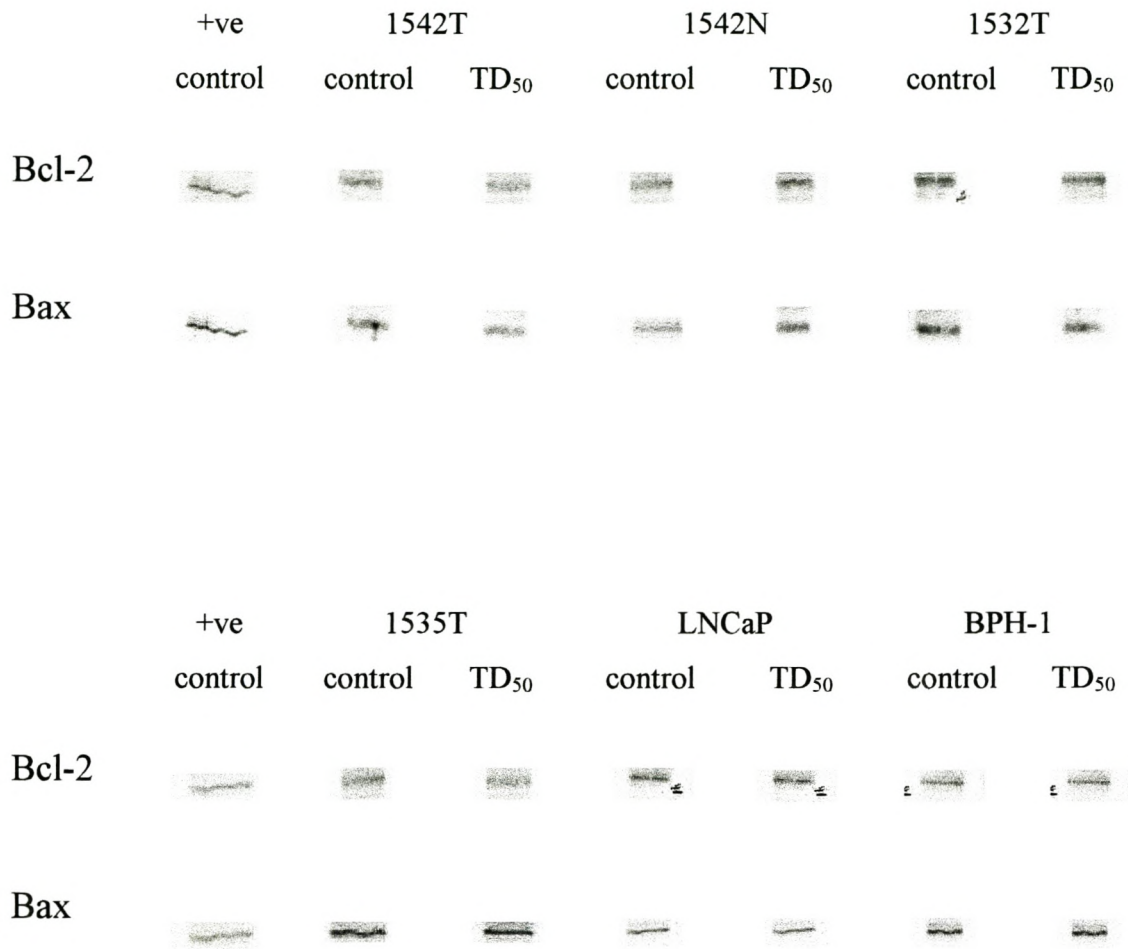
Figure 17: Western blot analyses of apoptotic-related protein expression pre- and post-TD_{50} etoposide treatment, in a panel of six prostate cell lines. The bladder carcinoma cell line, RT4, served as the positive control.
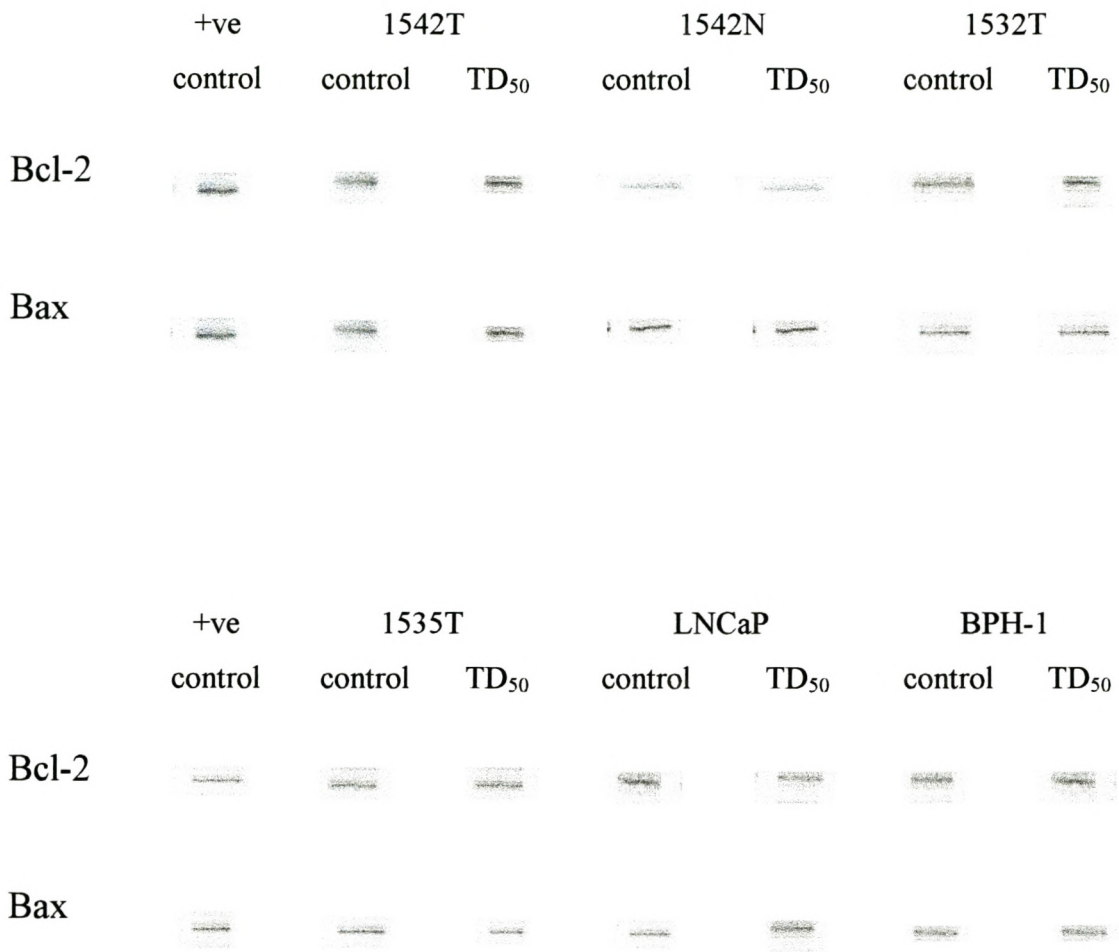
Figure 18: Western blot analyses of apoptotic-related protein expression pre- and post-TD$_{50}$ vinblastine treatment, in a panel of six prostate cell lines. The bladder carcinoma cell line, RT4, served as the positive control.
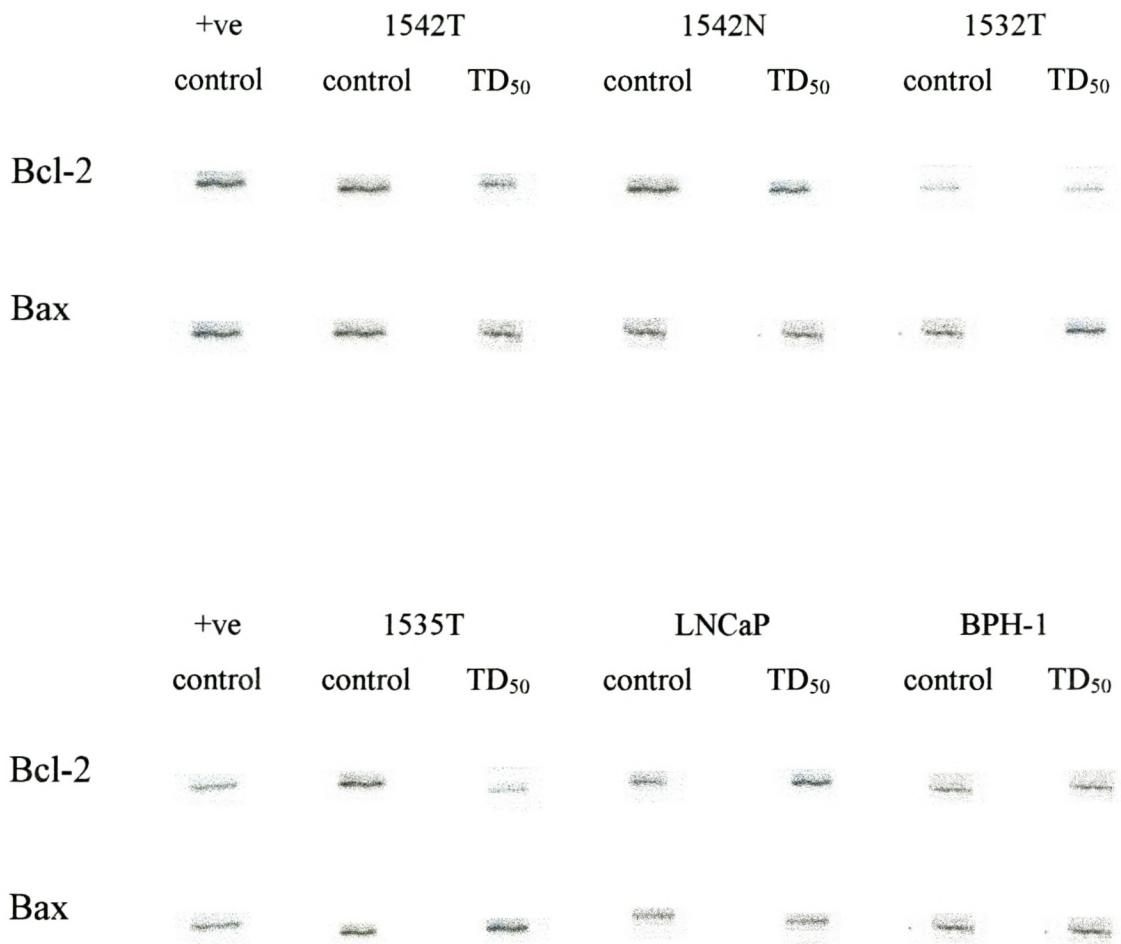
Figure 19: Western blot analyses of apoptotic-related protein expression pre- and post-TD$_{50}$ estramustine treatment, in a panel of six prostate cell lines. The bladder carcinoma cell line, RT4, served as the positive control.

CHAPTER 4

DISCUSSION

1. Drug resistance in prostate cancer cell lines is influenced by androgen dependence and p53 status.

The human prostate cell lines, DU145, PC-3 and LNCaP, originate from metastatic lesions (Stone *et al* 1978, Kaign *et al* 1979, Horoszewicz *et al* 1983). These cell lines are in common use and represent the most widely used cell biological model. A model which is closer to clinical reality would be epithelial prostate cultures which reflect the *in situ* characteristics of normal epithelium, the epithelium of benign hyperplasia, and the malignant epithelium (Hayward *et al* 1995, Bright *et al* 1997). Cell lines derived from normal, BPH and cancer tissue were chosen to cover the range from non-prostatic disease through to prostatic cancer. The possibility that prostate cancer may arise from prostatic hyperplasia is still controversial (Kirby *et al* 2001). Prostate cancer most commonly arises within the peripheral part of the prostate, while BPH predominantly develops within the transition zone. However, it has been reported that patients with BPH have a much greater risk of developing prostate cancer than men without BPH (Armenian *et al* 1974). On the other hand, there is no clear evidence that BPH is a direct precursor of adenocarcinoma (Kirby *et al* 2001). Nevertheless, a cell line such as BPH-1 can serve as an acceptable model for the transformation of a non-tumorigenic prostatic epithelium to a tumorigenic phenotype.

The cell lines grow in the absence of foetal bovine serum (FBS), with or without 5α-dihydrotestosterone (DHT) supplementation, at rates comparable to that in the presence of serum. It is apparent in Figure 7 that LNCaP cells respond to DHT stimulation and hence are androgen sensitive. This is consistent with published data (Horoszewicz *et al* 1983). It is also evident that all the transformed cell lines are androgen independent. The differences in growth between the FBS-cultures and the charcoal-stripped (cs)FBS-

cultures may be attributed to the removal of substances, other than steroids, which are essential for growth. This is in agreement with the observation that the addition of the synthetic androgen, methyltrienolone (Bright *et al* 1997) can restore cell growth after charcoal stripping of the growth medium. The rationale for determining the hormone status was to confirm that androgen sensitivity was not lost in culture. Janssen and colleagues elaborated on this problem (Janssen *et al* 1995).

As expected, cell inactivation, measured by crystal violet assay, was drug and concentration dependent (Figure 9, Table 2). The crystal violet assay is well established to measure the toxicity of radiation and chemotherapeutic drugs. Using tritiated thymidine to inactivate background cell population, the surviving fraction of established cell lines measured over a dose range of 0-6Gy by the standard clonogenic assay is in close agreement with results obtained with the crystal violet assay (Baker *et al* 1986). The differential response of cell lines to the same drug could be due to multidrug resistance (MDR). The following patterns can be distinguished: the 1542T cell line is the most chemosensitive, and the LNCaP cell line is the most chemoresistant. I speculate that the greater chemosensitivity of LNCaP cells against estramustine, as compared to BPH-1 cells, may be due to an androgen-suppressing effect of this drug. Interestingly, the 1542N cell line appears more resistant to vinblastine, etoposide and estramustine, than its tumour counterpart, 1542T (Figure 9). Unlike Berchem *et al* (1995) and Raffo *et al* (1995) the transition of prostate cancer cells to a hormone independent state was not found to generate chemoresistance. The results clearly demonstrate that the hormone independent cell lines are the most chemosensitive (Figure 9, Table 2). Estramustine proved to be the least toxic agent. This is in agreement with other studies showing that estramustine has no significant single-agent activity in hormone-refractory prostate cancer (Speicher *et al* 1992, Pienta *et al* 1996). However, there is *in vitro* evidence that a combination of estramustine and taxol, or estramustine and etoposide, has a supra-additive cytotoxic effect on prostate cancer cells (Speicher *et al* 1992, Pienta and Lehr 1993). Clinical studies have now demonstrated the efficacy of these combinations in phase 2 trials using estramustine plus vinblastine, etoposide, docetaxel and paclitaxel (Dimopoulos *et al* 1997, Smith *et al* 1999, Petrylak *et al* 1999).

In the LNCaP cell line it has been demonstrated that androgens are capable of inducing resistance to chemotherapy in a bcl-2-dependent manner (Berchem *et al* 1995). In a previous study in a panel of prostate cell lines I have shown that chemoresistance is linked to androgen dependence (Serafin *et al* 2001).

Drug-induced cell inactivation often invokes programmed cell death or apoptosis (Reed 1997). Model systems have demonstrated that factors which influence the apoptotic pathway can confer relative resistance to cytotoxic agents (Kamesaki 1993). In this scenario the apoptosis inhibiting bcl-2 gene product (Eliopoulos *et al* 1995, Reed 1997, Mackey *et al* 1998) would be of interest as a role player in cell toxicity. A major factor in the cellular damage response is the p53 tumour suppressor gene. Evidence for a link between the growth suppressing activity of p53 and the inactivation of cyclin-dependent kinases has been provided by the cloning of the WAF1/CIP1 gene (p21), the transcription of which is directly activated by p53. Expression of WAF1/CIP1 in mammalian cells inhibit cell growth, suggesting that p21 is a downstream mediator of p53 function (El-Deiry *et al* 1993). It has also been shown that WAF1/CIP1 is induced by DNA damaging agents that trigger G_1 arrest or apoptosis in cells with wild-type p53 but not in tumour cells where p53 is mutated or deleted (El-Deiry *et al* 1994). Michieli *et al* (1994) have suggested the existence of two separate pathways for the induction of WAF1/CIP1, a p53-dependent one elicited by DNA damage which leads to growth arrest, and a p53-independent one triggered by growth factors associated with cell growth. It is clear, therefore, that p53 status is important and that factors which influence the functionality of this gene need to be considered.

The establishment and maintenance of stable human prostate epithelial cell lines from primary tumours has met with difficulties in the absence of *in vitro* immortalisation (Schwab *et al* 2000). The 1532T, 1535T, 1542T, 1542N and *BPH-1 cell lines were immortalised by transduction with a recombinant retrovirus encoding the E6 and E7 genes of human papilloma virus serotype 16 (HPV-16), or the *large T antigen gene of simian virus 40 (SV40). Expression of the E6 gene results in cellular immortalisation through inactivation of the p53 growth suppressor protein, whereas expression of E7 destabilises retinoblastoma (Rb) protein, and activates telomerase to maintain telomeres in the chromosomes (Coursen *et al* 1997, Somasundaram 2000).

The SV40 large T antigen, in contrast, also abrogates the p53 and Rb pathways, mediating both cellular immortalisation and transformation (Martin and Chou 1975). The ability of SV40 large T antigen to induce transformation may be due to mutations in other cellular genes not directly effected by the T antigen (Ozer *et al* 1996). The transformation procedure may affect certain aspects of the cell phenotype, such as the proliferation rate (doubling time), the ability to grow without serum or androgen stimulation, the formation of colonies in soft agar, and the formation of tumours *in vivo* (Schwab *et al* 2000). It is, therefore, not surprising that only the non virus-transformed cell line, LNCaP, expressed functional p53 as indicated by the induction of p21 (Figure 8).

Recent data on Wi38VA13 cells have shown that p53 can be functional in certain types of SV40 transformed cells (Werner *et al* 2001). An important conclusion of the p21 data is that all virus-transformed cell lines (BPH-1, 1532T, 1535T, 1542T, and 1542N) show complete abolition of p53 function and not partial p53 inactivation by incomplete complexation to the virus product.

The conclusion of the p21 controls and p53 functionality tests are that the p53-defective cell lines emerge with superior sensitivity to vinblastine and etoposide. This is evident from the low TD_{50} data for etoposide and vinblastine (Table 2) which demonstrate that the p53-defective cell lines are approximately 10 times more drug sensitive than the LNCaP line in which p53 is intact.

The finding of superior drug sensitivity in p53 defective prostate cell lines is in contrast to results on human colon cancer cell lines which have shown that p53 disruption rendered cells resistant to the antimetabolite, 5-fluorouracil (Bunz *et al* 1999). However, a definitive conclusion of the influence of the p53 degrading viral proteins is not possible because it appears that the E6 protein has effects other than those mediated by p53 inhibition (Klingelhutz *et al* 1996).

The androgen sensitive tumour line, LNCaP, emerges as 10 times less sensitive to vinblastine and etoposide. It is furthermore indicated that estramustine does not distinguish benign hyperplasia cells from prostate tumour cell lines. Regrettably, most of the cell lines investigated here were androgen independent. Availability of a

p53-defective androgen dependent tumour cell line could have expanded the drug sensitivity assessment even further.

2. Studies on the influence of DNA repair on radiosensitivity in prostate cell lines.

Ionising radiation induces various types of DNA damage, such as double-strand breaks (dsb), single strand breaks (ssb), base damage and DNA protein crosslinks (Arrand and Michael 1992). The major factors determining radiosensitivity in human tumour cell lines are DNA dsb induction and repair (Ruiz de Almodovar *et al* 1994, Nunez *et al* 1996, Karran 2000). Differences in DNA repair proficiencies are widely known to affect cellular radiosensitivity (Hu and Hill 1996, Britten *et al* 1997, Dolling *et al* 1998).

Using six prostate cancer cell lines, varying in radiosensitivity from a mean inactivation dose of 1.62 - 2.77Gy, I show that the initial level of DNA damage per unit dose is cell line dependent. This is consistent with other data showing that the induction of DNA dsb varies widely between cell lines (Smeets *et al* 1993, Ruiz de Almodovar *et al* 1994, Roos *et al* 2000). Some studies have suggested that no correlation exists between radiosensitivity and repair of double-strand breaks (Smeets *et al* 1993, Olive *et al* 1994). High dose work (0-100Gy) on AT2BE, NF, R1H, CHO, CHO-K1, xrs1 and xrs5 human and rodent cell lines, has demonstrated a relationship between radiosensitivity and the number of unrepaired dsb (Smeets *et al* 1993, Dikomey *et al* 1998). At low dose (0-10Gy), radiosensitivity correlates with the initial DNA damage (Roos *et al* 2000). The absence of unrepaired DNA dsb measured by the constant field gel electrophoresis (CFGE) assay gives no information about the quality of the repaired DNA because mutations and misrepair are not detected by the CFGE method and will feature as repaired DNA (although loss of clonogenicity may ensue later). I demonstrate here for 6 prostate cell lines that a good correlation exists between the 2 hour DNA repair and radiosensitivity, and that the more radioresistant cell lines show better repair competence (Figure 12). No

dependence of radiosensitivity on 20 hour DNA repair could be demonstrated (Figure 13).

Since the majority of the cell lines were androgen independent there was little point in conjecturing about the role of androgen dependence in the correlation between radiosensitivity and repair. The hormone sensitive LNCaP line fits the 2 hour repair correlation. When the LNCaP data was removed from the plot, it emerged that the 2 hour correlation still applied, albeit at a lower correlation coefficient ($r = 0.88$ and $p = 0.052$), whereas the 20 hour correlation deteriorated ($r = 0.550$ and $p = 0.337$). DNA repair consists of a fast component (2-3 hours) during which the bulk of the DNA is repaired by non-homologous end-joining (NHEJ), and a slow component which is completed 16-24 hours after irradiation (Dikomey *et al* 1998), therefore 2 hour and 20 hour time points were chosen to examine the correlation between DNA repair and radiosensitivity. In the 6 prostate cell lines studied here, the G_2 block maximum is reached 12-42 hours after irradiation, and an additional 24-60 hours plus are required for the G_2 block to resolve. While G_2 checkpoint activation and repair may be interrelated, a definite requirement of a G_2 block as a repair facilitator is not obvious. My findings are at variance with results on a variety of rodent and human cell lines which show that radiosensitivity is correlated with 20 hour DNA repair (Dikomey *et al* 1998, Theron *et al* 2000). Within the limitations of a cell biological model this highlights the different behaviour of prostate tumour cells in terms of recovery from DNA damage. That recovery from DNA damage can be successfully exploited by drugs has been demonstrated for DU145, BM1604 and LNCaP cells which show a markedly enhanced sensitivity to cisplatin, vinblastine and etoposide, when the drugs are given under conditions of G_2 block abrogation (Serafin *et al* 2001). This staging of irradiation to produce a G_2 block followed by cytotoxic drugs given with a non-toxic G_2 block abrogator (Pentoxifylline) forces cells into mitosis and hence to change repair mode from homologous recombination (HR) in G_2 to non-homologous end-joining (NHEJ) at G_1. Results on caffeine, which is closely related to pentoxifylline, have now demonstrated that the HR repair process is specifically inhibited (Asaad *et al* 2000).

DNA damage induces the p53 tumour suppressor protein (Lee and Bernstein 1995). When wild-type p53 cells are damaged by irradiation the increase of p53 protein

causes a G_1 arrest and cells enter repair or apoptosis (Lee and Bernstein 1995). P53 mutant cells present in the majority of tumours show a G_2 cell cycle arrest which facilitates DNA repair and prevents the propagation of the defective genome to daughter cells (Maity *et al* 1997). The downstream effects of p53 have lead to contradictory hypotheses regarding the influence of p53 on radiosensitivity (Bristow *et al* 1996). For example, p53 wild-type cells could initiate apoptosis leading to cell death, and cells also could show a G_1 and/or G_2 arrest and increase of DNA repair leading to increased cell survival. P53-dependent apoptosis and G_2 arrest, followed by DNA repair and cellular recovery, appear to be controlled by different pathways which operate differently between cell types (Morgan and Kastan 1997). Except for the p53 wild-type LNCaP cells (Horoszewicz *et al* 1983), the cell lines used here were immortalised with the large T-antigen of the simian virus 40 (BPH-1) (Hayward *et al* 1995), and with the E6 and E7 genes of the human papilloma virus 16 (1532T, 1535T, 1542T and 1542N) (Bright *et al* 1997) and thus are p53 dysfunctional.

My results are in agreement with the general notion that cell death mainly results from DNA double-strand breaks (Frankenberg-Schwager 1989). Interestingly, however, it is apparent for this range of prostate cell lines that cellular radiosensitivity correlates with the number of dsb measured within 2 hours of irradiation. Results on head and neck squamous carcinoma tumour cells, HNSC, SCC, JSQ, and SQ also show this correlation (Schwartz *et al* 1988). The fact that the 2 hour repair period can separate radiosensitive from radioresistant cells in 2 groups of human tumour cell lines has implications for therapy, and would be consistent with the clinical observation that prostate tumours can be successfully controlled by low dose rate-brachytherapy (Raqde *et al* 1998).

3. The influence of p53 and bax/bcl-2 on chemosensitivity in benign and malignant prostatic cell lines.

P53 status is an important determinant of tumour responsiveness to antineoplastic agents (Lowe 1995, Chresta *et al* 1996, Wilson *et al* 1997). I have assessed the role of p53 and bax/bcl-2 in drug- and irradiation-induced apoptosis in 6 prostate cell lines. It has been noted that loss of p53 function leads to resistance to chemotherapy (Weller 1998, Keshelava *et al* 2001), and to radiation therapy (O'Connor *et al* 1993). I determined p53 function by measuring induction of p21 in the prostate cell lines in response to 7Gy ^{60}Co γ-irradiation. The p53-inactivated cell lines, 1532T, 1535T, 1542T and 1542N, are shown to exhibit enhanced sensitivity to etoposide and vinblastine (to a greater degree), and estramustine (to a lesser degree), compared with the p53 wild-type cell line, LNCaP. The BPH-1 cell line responded well only to etoposide. This result is in agreement with research showing that inactivation of p53 enhances sensitivity to multiple chemotherapeutic agents (Hawkins *et al* 1996, Wahl *et al* 1996). There may be several explanations for the conflicting results. Immortalisation of cell lines with human papilloma virus (HPV-16) E6 and E7, and simian virus 40 large T-antigen (SV40) results in inactivation of p53 (Martin and Chou 1975, Morales *et al* 1999). This complicates the interpretation of experiments on drug sensitivity because HPV-16 E6 has effects on cells other than those mediated by p53 inhibition (Klingelhutz *et al* 1996). Furthermore, tumour cells may acquire additional genetic abnormalities in the process of becoming immortal (Masters 2000) that result in drug resistance. The role of p53 in drug sensitivity could also be dependent on the class of chemotherapeutic used. Finally, certain cell types may display a predominant apoptotic response to chemotherapeutic agents, and this could explain their relative sensitivity to drugs. Fan and colleagues showed enhanced sensitivity to drugs in breast cell lines which express HPV-16, yet do not have a predominant apoptotic response (Fan *et al* 1995).

The normal function of p53 includes cell cycle arrest at the G_1 and/or G_2 checkpoint in response to DNA damage to facilitate DNA repair and cell recovery (Kastan *et al* 1991). P53 mutant, but not p53 wild-type, melanoma and squamous cell carcinoma cell lines have been shown to undergo dramatic chemosensitisation under conditions

of G_2 block abrogation (Binder *et al* 2000). Similar enhancements of drug sensitivity have been demonstrated for p53 mutant prostate cell lines when the G_2 block is abrogated (Serafin *et al* 2001). The results show that early entry into mitosis renders these cells particularly drug sensitive. Recent work on the repair response of irradiated XRCC2 and XRCC3 cells defective in HR-repair has highlighted the increased sensitivity of HR repair in G_2 to caffeine (Asaad *et al* 2000). In the p53 wild-type cells the proportion of G_2-blocked cells which enter early mitosis is usually lower than 50%. This means that only a smaller G_2 population (approximately 20%) enters the G_1 phase. The lower toxicity enhancements observed in p53 wild-type cells may be due to the lower population of G_2-blocked cells (Serafin *et al* 2001). Activation of p53 can also induce apoptosis, and several studies have linked wild-type p53 with susceptibility of tumour cells to DNA-damaging anticancer agents (Lowe *et al* 1994, Wilson *et al* 1997). The conflicting reports on the influence of p53 status on chemosensitivity raises the possibility that different chemotherapeutic agents may have different p53-dependent effects in different tumour cells (Mueller and Eppenberger 1996).

Data from clinical studies demonstrate that tumour burden affects patient performance; the greater the tumour burden, the less likely the response to treatment (Crawford *et al* 1999). Thus, it may be beneficial to start chemotherapy early in the disease. The slow development of prostate cancer may offer opportunities for early application of chemotherapy, including patients with a rising PSA level (Smith *et al* 1999). However, it is generally agreed that chemotherapy is not effective in the treatment of primary prostate cancer (CF Heyns, personal communication).

Evidence obtained in the last few years has established that cytotoxic effectiveness is related to the ability of drugs to induce apoptosis (Hannun 1997). The apoptotic program is controlled by a variety of genes, many of which are mutated and/or dysfunctional in human cancers (McGill and Fisher 1997). An important example is the p53 tumour suppressor gene and members of the bcl-2 gene family (Reed *et al* 1996, McGill and Fisher 1997). Exposure to etoposide, vinblastine and estramustine, and morphological scoring of apoptosis after acridine orange staining, shows that neither normal nor malignant prostate cells exhibit high levels of apoptosis at any time point up to 48 hours. An increased level of apoptosis was observed in the malignant

cells only for the drugs, etoposide and vinblastine. Similar results were obtained for the same panel of cells at ^{60}Co γ-irradiation doses which result in 50% clonogenic survival, in the 0-48 hours time window. Many groups have indicated that apoptosis may be the primary mode of death following gamma-irradiation only in specific cell types such as haematopoetic or lymphocytic cells, but not in stromal- or epithelial-derived tissues (Kyprianou *et al* 1997, Tannock and Lee 2001, Sheridan and West 2001). Indeed, experiments using a new range of Ferrocene and Rhodium-Ferrocene drugs on the 1542 range of prostate cell lines indicate that necrosis (or late apoptosis), but not apoptosis, is the prime route of cell death induced by these drugs (Weber *et al*, unpublished data). Cisplatin, on the other hand, given under identical conditions elicits a marked apoptotic response (Weber *et al*, unpublished data). Akudugu *et al* have shown that the expression of apoptosis and micronuclei is influenced by the extent of DNA double-strand break repair within the first two hours after irradiation and that cell lines which repair more damage in the first 2 hours (by NHEJ) express more micronuclei and less apoptosis (Akudugu 2000). Attempts to alter apoptotic indices in epithelial- and stromal-derived tissues have failed to affect clonogenic survival, suggesting that whilst the apoptotic pathway may be intact in malignant prostate cells, other death mechanisms may override this response following irradiation (Bromfield *et al* 2003).

Since the apoptotic levels for both p53-functional and p53-non functional cell lines were low, it is difficult here to make a case for p53 functionality in apoptosis. My results are at variance with reports that human papilloma virus E7 renders cells dramatically more sensitive to apoptosis in response to various genotoxins (Brown and Wouters, unpublished data). It has been argued that p53 is not strictly required for drug-induced cell death because at high drug doses all anticancer drugs induce apoptosis (and other types of death) independently of p53. In fact, the contribution of p53 to drug-induced apoptosis is influenced by a variety of factors, such as the nature of the toxin, dose, tissue and mutational background of the tumour (Bunz *et al* 1999, Wallace-Brodeur and Lowe 1999).

Susceptibility to apoptosis is modulated by a family of proteins that have homology to conserved regions of the bcl-2 gene (Yin *et al* 1994). Apoptosis is promoted by some of the bcl-2 homologues (bax, bak, bad, and bclX$_s$) (Farrow *et al* 1995, Yang *et al*

1995), whereas other members of the bcl-2 family (bcl-2, bclX$_L$, and bag-1) form dimers with the apoptosis-promoters and inhibit their activity, hence suppressing apoptosis (Reed 1994, Takayama *et al* 1995). It has been suggested that a high bcl-2/bax ratio may be a useful prognostic indicator for chemoresistance (Pepper *et al* 1997). In my own immunoblotting experiments no changes were detected in the expression of either bax or bcl-2 in the six prostate cell lines, after etoposide, vinblastine and estramustine drug treatment. This is in agreement with the findings of Chresta *et al* (Chresta *et al* 1996), but differs from the data of Zhan *et al* (Zhan *et al* 1994). It is speculated that increases in bax and bcl-2 are cell type-specific.

It has been stated that much of the published evidence relating to the role of apoptosis following treatment with radiation or anticancer drugs is indirect (Tannock and Lee 2001). The presence of various apoptotic markers are noted, and it is assumed, implicitly or explicitly, that a cause and effect relationship exists between the expression of these markers and loss of cell viability (Tannock and Lee 2001). Some have found a positive relationship (Kramer *et al* 1996, Tai *et al* 1998), and others have not (Pereira *et al* 1997, Veronese *et al* 1998). The results presented here suggest that apoptosis is not a major route of drug- or radiation-induced cell death in prostate cell lines. From the kinetics in the 0-48 hours time window it appears unlikely that increasing the post-treatment observation time to 96 hours (when the earliest surviving clonogens can be detected) will reveal a correlation between reproductive cell death and apoptosis. Indeed, several investigators have shown that changes which affect the expression of genes that control apoptosis have no influence on clonogenic survival (Kyprianou *et al* 1997, Beale *et al* 1998, Tannock and Lee 2001).

The major findings of this research are that the extent of apoptosis in the loss of cell viability following anticancer drug or irradiation treatment is cell line- and drug-dependent, and that the overall level of apoptosis induced by the three established cytotoxic agents and ^{60}Co γ-irradiation is rather low. It therefore appears that major cellular deterioration in prostate cells is not coupled to apoptosis. This is not entirely surprising because high levels of apoptosis are usually associated with high cell turnover such as cells of the lymphatic system. My conclusions are entirely consistent with other recent findings (Brown and Wouters 1999, Tannock and Lee 2001, Steel 2001, Bromfield *et al* 2003) strongly suggesting that neither the p53 status nor the

ability of the cells to undergo apoptosis plays a significant role in the sensitivity to DNA-damaging agents.

4. Clinical implications of findings, and possible future avenues for research.

Understanding of the role of apoptosis and other death pathways is of great importance for cancer treatment. If anticancer drugs and radiation damage give rise to either cell death or repair it would be of clinical interest to identify agents which would select these pathways. For example, inhibition of the bcl-2 protein, or up-regulation of the bax protein, might be used to increase killing of tumour cells. Conversely, up-regulation of bcl-2 or other genes that protect against apoptosis might be an important target for control of damage to normal tissue.

The success of radiation therapy in the treatment of prostate cancer would depend upon the eradication of all tumour clonogens (ie. tumour stem cells) to suppress unlimited cell proliferation. Failure to eradicate these clonogens will result in the re-growth of the tumour after treatment (Wong and Hill 1998). The mode of clonogenic cell kill following DNA damage would be important in defining new strategies for the control of prostate cancer and the understanding of the processes following radiotherapy. Clinico-pathologic studies have not succeeded to correlate altered expression of the p53, p21, bax, bcl-2, and caspase apoptosis-related genes to radiocurability (Rakozy et al 1998). This could be due to the existence of alternative pathways of cell death which obscure analyses focussed on apoptosis (Akudugu and Bohm 2001, Abend et al 2000). There is increasing evidence that apoptosis is not the sole mode of cell death following radio- and chemotherapy in epithelial tissues (Dewey et al 1995, Olive et al 1999, Steel 2001). Indeed, it has been suggested that apoptosis may actually occur in a non-clonogenic population after DNA damage (Tannock and Lee 2001), and that a permanent cell-cycle arrest or senescence-like terminal growth arrest may also be a factor in determining prostate cell death

following radiotherapy (Schwarze *et al* 2001). A comprehension of the range of death responses of prostatic tumour cells clearly is important for therapy as it sets event markers which could be indicators of the success of cell inactivation.

Because inhibitors of Poly(ADP-ribose) polymerase (PARP) are able to potentiate the cell killing effects of some DNA-damaging agents and to inhibit repair of induced DNA strand-breaks, such compounds may enhance the anti-tumour efficacy of radiotherapy or cytotoxic drug treatment. PARP is known to regulate chromatin structure by poly-ADP-ribosylation of nuclear proteins, to facilitate DNA base excision repair, and to contribute to cellular recovery. Using a PARP-inhibitor, a 30-50% increase in radiosensitivity has been observed *in vitro* in human prostate cancer cell lines (Schlicker *et al* 1999). It is conceivable that such agents could be effective against prostate tumours and it may be worthwhile to investigate their efficacy in prostate cell lines.

CONCLUSIONS

The human prostate cell lines 1532T, 1535T, 1542T, 1542N, BPH-1 and LNCaP were used to assess the influence of p53 status, p53 functionality, androgen dependence, DNA repair, apoptosis and apoptosis-related proteins in response to DNA damage induced by chemotherapeutic drugs and gamma irradiation.

The results demonstrate that loss of p53 function and androgen independence is not correlated with cellular resistance to vinblastine, estramustine and etoposide. The p53 functionally-inactive cell lines were also androgen independent, and are shown to be the most sensitive to the three anticancer drugs. While this observation is at variance with published work suggesting that p53 mutants are resistant to treatment, it may well be that the effects observed here are cell line- and drug-dependent.

The six prostate cell lines were found to show differences in radiosensitivity. When radiosensitivity expressed as mean inactivation dose ($\overline{D}$) was compared with residual DNA damage remaining after 2 hours and 20 hours of repair, it was found that radiosensitivity is significantly correlated with the 2 hour DNA repair component, showing a correlation coefficient of 0.92 (p = 0.009). The more radioresistant cell lines were found to show better repair competence. Since only one of the human prostate cell lines was androgen sensitive, no case could be made for the influence of androgen on radiosensitivity and DNA repair capacity.

Analysis of the role of p53 and bax/bcl-2 in drug- and irradiation-induced apoptosis suggests that neither the p53 status nor the ability of the cells to undergo apoptosis plays a significant role in the sensitivity to DNA-damaging agents. The overall level of apoptosis for both p53-functional and p53-non functional cell lines in response to the three cytotoxic agents and ^{60}Co γ-irradiation was found to be in the range of 0.1-12%, and is rather low. While it cannot be excluded that the apoptotic pathway operates in malignant prostate cells, other death mechanisms may override this response. The level of apoptosis in response to drug or irradiation treatment was

found to be cell line- and drug-dependent. Immunoblotting failed to detect any changes in the expression of either bax or bcl-2 in all six prostate cell lines after treatment with etoposide, vinblastine and estramustine, and both proteins were expressed at a basal level.

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