CHEMOSENSITIVITY OF PROSTATIC TUMOUR CELL LINES UNDER CONDITIONS OF G2 BLOCK ABROGATION

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Dissertation submitted in fulfilment of the requirements for the Master's Degree in Technology (Biomedical Technology) in the Faculty of Applied Sciences at the Cape Technikon.

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JOHN PETER LOWE
"A MAN FOR ALL SEASONS"

DECLARATION

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

25.10.2000

Signature: Date:

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ABSTRACT

Cancer of the prostate gland is now recognised as one of the principal medical problems in males. In the USA, cancer of the prostate is the second most commonly diagnosed cancer after skin cancer and the second most common cause of death from cancer after lung cancer. In South Africa, prostate cancer is the second most common cancer, with an estimated annual incidence of 19.1 per 100 000 men (Sitas, 1994). However, this incidence is probably under-estimated, due to incomplete records. Comparison of the incidence of prostate cancer in the different racial groups shows that it is the second most common malignancy in the White, Black (African) and Mixed (Coloured) race groups, and the fourth most common malignancy in Asian (Indian) men in South Africa.

Metastatic prostate cancer is refractory to hormone therapy and remains incurable. Hence, novel therapeutic approaches are needed. These anticancer drugs can be tested in tumour cell lines, and cell culture methods also permit testing of optimum conditions.

Evidence in squamous cell carcinoma and melanoma cells have shown that the toxicity of established anticancer drugs can be markedly enhanced when the drug is added under conditions of G_2 block abrogation (Binder *et al*, 2000). In this study I show that this approach is also highly effective in the prostate cancer cell lines, DU145, BM1604 and LNCaP. The cells were irradiated with 7Gy ^{60}Co γ-irradiation and when the G_2 block was maximally expressed, Cisplatin, Etoposide and Vinblastine, at a toxic dose of 10% (TD_{10}), were added with 2mM pentoxifylline, and cell survival determined by colony assay. It was found that enhancement factors were greater in the TP53 mutant cell lines, DU145 and BM1604, than in the TP53 wild-type cell line. The response of the TP53 wild-type cell line, LNCaP, was weak for all three drugs, showing enhancement factors (EFs) of 1.10, 1.21, and 1.47 for Etoposide, Vinblastine and Cisplatin, respectively. When the drug was added 8 hours after maximum G_2 block expression, the enhancement factors were found to be

1.50, 1.38 and 1.57 for Etoposide, Vinblastine and Cisplatin, respectively.

In the TP53 mutant cell lines, DU145 and BM1604, EFs were found to be 3.30 and 3.60 respectively, for Cisplatin, 2.30 and 1.53 respectively, for Vinblastine, and 2.40 and 4.00 respectively, for Etoposide, when the drugs were added at maximum G_2 block expression.

When the drugs were added 8 hours after maximum G_2 block expression, EFs were found to be 4.11 and 4.50 respectively, for Cisplatin, 4.82 and 2.60 respectively, for Vinblastine, and 1.50 and 1.00 respectively, for Etoposide.

The observed sensitisation of prostate cancer cells accomplished by this method offers a possibility of selecting particular radiation resistant TP53 mutant prostate tumours for more effective therapy. The fact that the enhancements are observed at very low toxic drug doses would allow for sparing of normal tissue.

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LIST OF ABBREVIATIONS

OD	Optical density
PI	Propidium iodide
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
SF	Survival fraction
SF2	Survival fraction at 2Gy
SSD	Source-to-sample distance
REF	Radiotoxicity enhancement factor
EF	Dose enhancement factor
PFA	Paraformaldehyde
BSA	Bovine serum albumin
Ab	Antibody
IgG	Immunoglobulin
FITC	Fluorescein isothiocyanate
PTX	Pentoxifylline
SCC	Squamous cell carcinoma
dsb	double-strand breaks

INTRODUCTION

Prostate cancer – the clinical problem

Prostate cancer is the second leading cause of male cancer deaths and the most commonly diagnosed cancer in men in the United States of America (Lynn and Ries, 1995). It 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*, 1993). 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 the 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.

Aetiology and Pathogenesis

Despite the increasing incidence of prostate cancer, particularly in Western countries, the aetiology and pathogenesis remain unclear. Classic epidemiological studies have implicated a number of

aetiological factors (eg. a family history of prostate cancer, a high saturated fat intake, a low intake of carotenoids, high serum androgen levels, and other factors) (Ekman *et al*, 1999).

Several small studies have shown some evidence of familial clustering of prostate cancer cases, using selected groups (Cannon-Albright *et al*, 1994). In addition, there are many problems associated with the conduct and interpretation of retrospective dietary studies. For instance, the accuracy of recall may be suspect, or the current diet may be less relevant to a disease process which may have been initiated many years previously. There is, therefore, a need for epidemiological studies to be more representative of the whole population of prostate cancer patients, rather than subgroups!

Multiple steps are required to produce clinical prostate cancer. It is possible that men inherit a gene or genes predisposing them to early development of prostate cancer and that environmental factors result in further genetic damage. The prostate is continuously exposed to androgens which stimulate cell proliferation. It has been proposed that androgens form a final common pathway in the action of several aetiological factors, particularly dietary fat intake (Ross and Henderson, 1994).

Evidence exists to support the idea of a "two-disease" theory of prostate cancer : young patients with a strong inherited predisposition in whom dietary and other environmental factors have little influence ; and more elderly patients in whom non-inherited factors play a greater role (Zumoff *et al*, 1982).

Androgen independence

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.

Despite their androgen independence, most prostate tumours express high levels of androgen receptors in the nuclei of the tumour cells. It has long been speculated, therefore, that the androgen receptor gene could play a role in the initiation or progressive growth of androgen-independent prostate tumours (Tilley *et al*, 1996).

PSA screening

Because of the poor prognosis for men with metastatic prostate cancer, aggressive screening programmes have been suggested for men starting at the age of 50, to permit the early detection of prostate cancer while it is localised and potentially curable. The diagnosis of prostatic disease has been dramatically improved as a result of the discovery of the prostate specific antigen (PSA) (Lundwall and Lilja, 1987).

PSA is an organ-specific, kallikrein-like serine protease produced by prostatic epithelial cells lining the acini and ducts of the prostate gland. It is organ specific but not disease specific because it is expressed in both benign and malignant processes involving epithelial cells of the prostate. The PSA test has a sensitivity that ranges from 72-89% and a specificity between 59 and 91%. The combination of the digital rectal examination and the PSA is even more effective in the diagnosis of early prostate cancer.

Used intelligently, PSA is currently the most important, accurate and clinically useful tumour marker for prostate cancer. It has revolutionised the diagnosis, staging, management and follow-up of prostate cancer and it is regarded as the most useful tumour marker in oncology today. PSA has been in widespread clinical use since 1987. Since that time it has been used as a marker to measure

tumour recurrence after therapy and there have been many studies using it as a marker for early detection of prostate cancer (Oesterling, 1991).

Significant controversy surrounds the topics of screening for and the early detection of prostate cancer, and the PSA is central to this debate. The marked sensitivity of this marker deserves the enthusiasm it raises, but the issue of specificity raises some concerns. First, the lack of specificity (organ versus disease) of the PSA results in a considerable number of men without cancer undergoing biopsy. The associated cost, measured in financial and emotional terms, is significant. Second, at present there are no conclusive data to demonstrate a reduction in prostate cancer mortality resulting from early detection (Catalona *et al*, 1994).

Induction of programmed cell death by apoptosis-related oncoproteins

Apoptosis (programmed cell death) is involved in maintaining tissue homeostasis by ensuring that cell loss always equals cell gain by 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).

The mechanism whereby prostate cancer cells survive androgen ablation and become androgen independent is not entirely clear. Several different pro- and anti-apoptotic pathways exist and have been identified in androgen-independent prostate cell lines and tissues. Androgen-independent prostate cells maintain the ability to undergo programmed cell death (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 TP53 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 TP53 mutant, 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 TP53 tumour suppressor gene is the most frequently mutated gene found in multiple human malignant tumours, including prostate cancers. Several studies have shown TP53 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 TP53 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 TP53 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 TP53 and that multiple chemical entities induce apoptotic death of TP53-null PC3 cells (Borner *et al*, 1995) suggests that

TP53 may not be essential for the apoptotic response to occur in these situations (Berges *et al*, 1993). However, since TP53 has an important role in introducing the G_0/G_1 checkpoint, attenuation or loss of TP53 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.

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 should 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 providing 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.

Radical prostatectomy

Radical prostatectomy has emerged as the preferred surgical treatment for organ-confined ie. localised (stage T1 or T2) prostate cancer in patients with a life expectancy of 10-15 years or more (Vashi and Oesterling, 1999). It offers cancer cure rates superior to any other modality, and the complications can be managed successfully in the great majority of patients. Radical prostatectomy is recommended for younger patients with prostate cancer, and for patients with high-grade tumours. The patient wishing to preserve his potency will be better served with surgery if bilateral nerve-sparing is feasible. It is currently not known whether radiotherapy can control prostate cancer as effectively as radical prostatectomy, although the long-term data seem to favour surgical outcomes (Goluboff and Benson, 1996). Patients with clinical stage T3 prostate cancer are not considered to be ideal candidates for the radical prostatectomy – surgery alone cannot cure these patients.

Complete continence rates of 92-97% have been achieved with the radical retropubic prostatectomy, and preservation of potency stands at around 68% (Vashi and Oesterling, 1999).

Hormonal therapy

The treatment of metastatic prostate cancer has been straightforward since the landmark discovery of Huggins and Hodges in the 1940s that the disease is hormonally dependent and that castration brings significant palliation for advanced disease. Androgen withdrawal, androgen blockade, or combination therapy are current first-line treatment modalities (De Antoni and Crawford, 1994).

Although androgen ablation is standard therapy for metastatic prostatic cancer, this therapy is rarely curative. The major reason for this is that metastatic cancer within an individual is heterogeneous, including both androgen-dependent and -independent prostatic cancer cells, even before therapy is initiated (Tang and Porter, 1997). Thus, androgen ablation does not eliminate pre-existing

androgen-independent cells within the patient. Unfortunately, there are no currently utilised chemotherapeutic agents which can effectively control the growth of androgen-independent prostatic cancer cells. Thus, new approaches to controlling androgen-independent prostate cells are being sought.

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 PSA level less than 15ng/ml) (Porter and Hart, 1999), external beam radiation therapy offers a good chance of durable tumour control or eradication. 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 to gain, if anything, from surgical management of their prostate cancer. 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 (T2-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).

Brachytherapy is a form of radiation therapy that involves the implantation of radioactive sources into the prostate. This method of treatment can deliver more radiation to the prostate and a lower dose to the surrounding normal organs than can conventional therapy. Prostate brachytherapy is presently undergoing a resurgence with the advent of transrectal ultrasound transperineal

implantation. Three-dimensional dose distributions are calculated for the prostate, bladder and rectum in order to optimise the dose to the prostate while minimising the dose to normal tissues. The choice of isotope is based on the grade of tumour. Rigid guidelines have been described for patient selection : the cancer must be confined to the prostate, the prostate volume must be less than $60cm^3$, patients with severe obstructive symptoms are not implanted, T1 and early T2 tumours are treated with implant alone (Porter and Hart, 1999).

Cryotherapy

The fear of major surgery, the risk of urinary incontinence and/or impotence after radical prostatectomy, combined with uncertain benefit from treatment of localised disease, have led many patients and their physicians to seek other options for treatment.

Cryosurgical ablation is a minimally invasive surgical procedure performed under local anaesthetic. It's goal is destruction of the malignant and benign epithelium of the prostate by exposure to rapid freezing and thawing at extreme temperatures (Cohen *et al*, 1999).

Incontinence risks are minimal and comparable to those of external beam radiation therapy. Cryosurgical ablation has a risk of impotence similar to that reported for radical prostatectomy, since the treatment will extend out past the capsule, and thus will freeze the neurovascular bundles. Recovery of erectile function is usually limited to the younger population (Cohen *et al*, 1996).

Chemotherapy

Most all of the mortality and morbidity attributed to 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 (Greenlee *et al*, 2000). Options

for therapy in this setting include secondary hormonal therapy, chemotherapy, and experimental therapies.

The prevailing attitude for the past two decades that chemotherapy has little place in advanced prostate cancer stems, perhaps, from several factors : 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.

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 in prostate cancer, which stemmed from its poor efficacy, has been replaced with moderate enthusiasm within the urologic community. The last decade has witnessed a desire to investigate the potential benefits of chemotherapy in the treatment of prostate cancer. This has been fostered by the development of several new drug regimens with documented objective and 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 these regimens earlier in the course of the disease will, in the long run, have a more significant impact on the mortality of prostate cancer.

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). 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).

It was 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 trails. 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.

Chemosensitisers

Methylxanthines have been tested extensively in radiobiological research for their potential to increase the cytotoxicity of cells to radiation and chemotherapeutic drugs. Several studies have reported a dramatic enhancement of anti-tumour effects when chemotherapeutic drugs are combined with caffeine, but the clinical potential of caffeine has been severely limited by its neurological and cardiac toxicities (Dobmeyer *et al*, 1983). Recent studies have shown that another methylxanthine derivative, pentoxifylline, is a good dose modifying agent *in vitro* (Fingert *et al*, 1986). The chemical structures of pentoxifylline and caffeine are closely related and are given in

Figure 1. Unlike caffeine, pentoxifylline has gained wide clinical use because of lower toxicity in a variety of immune and vascular diseases (Aviado and Porter, 1984). In this study, pentoxifylline has been employed for its ability to influence cell cycle checkpoint control.

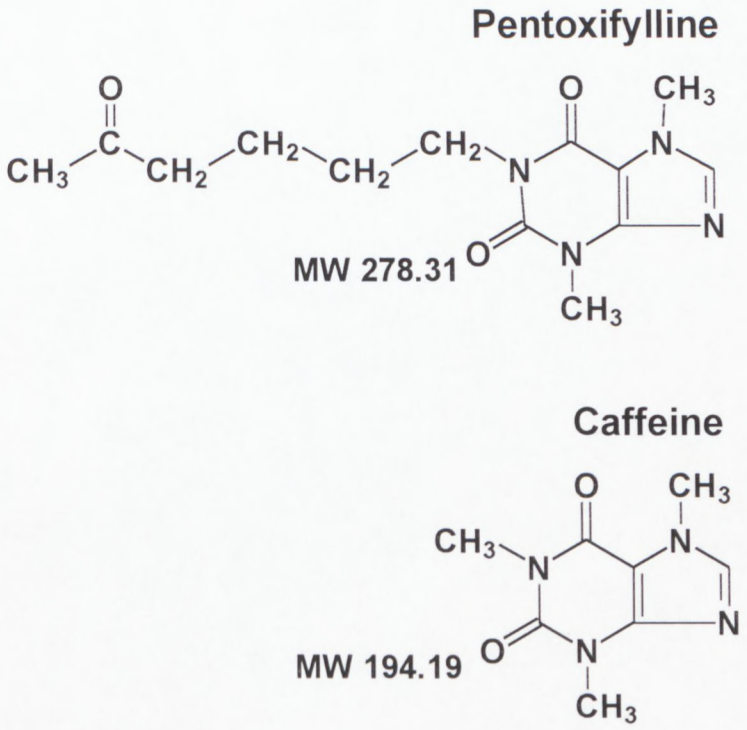

Figure 1: Chemical structures and molecular weights of the two closely related methylxanthines, pentoxifylline and caffeine.

The G_1S and G_2M (Figure 2) transitions are important checkpoints in the response of cells to irradiation and genotoxins. It is generally thought that delays in the G_1 or G_2 progression generate time for the cells to recognise and repair DNA damage, and to prevent the replication and propagation of defective genomes.

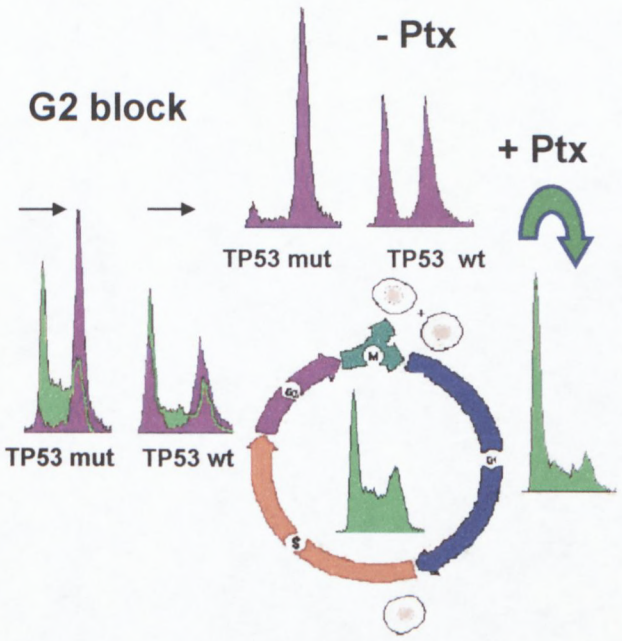

Figure 2: The cell cycle and DNA content of the cell.

Suppression of the G_2 checkpoint by pentoxifylline is an effective strategy whereby TP53 defective cells can be targeted for destruction by a second irradiation or chemotherapeutic insult (Binder *et al*, 2000). The molecular mechanism of G_2 block abrogation by pentoxifylline has not been fully elucidated but cyclin B1 and $p34^{cdc2}$ levels are rapidly restored after irradiation and facilitate early entry of G_2 cells into mitosis (Theron and Bohm, 1998, 2000).

Thesis objective

A consequence of premature entry of cells into mitosis (before repair) would be early demise and death because of retention of lethal lesions. It is clear, however, that early entry enforced by pentoxifylline is not lethal (because of rapid repair) and that dose modification of irradiation can only be enhanced when the pentoxifylline is added at the point of irradiation (Theron *et al*, 2000). Nevertheless, abrogation of the G_2 block can be harnessed for improved toxicity of irradiation when combined with a second challenge (Binder *et al*, 2000). In view of the fact that enhancement factors are in the region of up to 80, it would seem justified to test this approach in prostate cells. Such experiments could set the stage for real improvements to therapy.

The specific questions addressed in this thesis are:

(1) Can the chemosensitivity of prostate tumour cell lines be enhanced by the application of a low dose of drug in conjunction with a G_2 block abrogator?

(2) What is the time-dependence of this process?

(3) What is the role of TP53 status?

(4) What is the role of androgen status?

MATERIALS AND METHODS

Cell lines

DU145

The DU145 cell line was established from a metastatic lesion of the central nervous system. DU145 has an epithelial-like morphology, is adherent and grows as a monolayer in minimum essential medium (MEM) (Sigma, South Africa) supplemented with 10% heat-inactivated foetal bovine serum (FBS), penicillin (100U/ml) and streptomycin (100μg/ml). DU145 is TP53 mutant (Planchon *et al*, 1995), androgen independent and has a doubling time of 40 hours. The culture was obtained from Highveld Biological (Pty) Ltd.

BM1604

The BM1604 cell line was established from a localised human prostatic adenocarcinoma. BM1604 has an epithelial-like morphology, is adherent and grows as a monolayer in RPMI 1640 medium (Highveld Biological, South Africa) supplemented with 10% heat-inactivated FBS, penicillin (100U/ml) and streptomycin (100μg/ml). BM1604 is TP53 mutant, androgen independent and has a doubling time of 30 hours. The culture was obtained from Highveld Biological (Pty) Ltd.

LNCaP.FGC

The LNCaP cell line was established from a supraclavicular lymph node metastasis of human prostatic adenocarcinoma. LNCaP has a fibroblastoid morphology, low anchorage potential, is adherent but grows in aggregates and as single cells in RPMI 1640 medium (Highveld Biological, South Africa) supplemented with 5% heat-inactivated FBS, penicillin (100U/ml) and streptomycin (100μg/ml). LNCaP is TP53 wild-type (Planchon *et al*, 1995), androgen dependent and has a

doubling time of 36 hours. 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 culture was obtained from Dr JH Visser (Dept of Urology, University of Stellenbosch, Medical Faculty).

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

This vinca alkaloid, $C_{46}H_{58}N_4O_9$, is a microtubule inhibitor (PERIBLASTINE, Lennon Ltd.). Vinblastine is a cell-cycle-specific agent and blocks mitosis with metaphase arrest (Gilman *et al*, 1996). Each vial contains 1mg/ml of Vinblastine sulphate. Vinblastine has a molecular weight of 811.00

Cisplatin

This intercalating agent, $Cl_2H_6N_2Pt$, binds to DNA, and disrupts and unwinds the double helix (P&U CISPLATIN, Pharmacia & Upjohn Ltd.). There is no phase specificity in the action of Cisplatin on the cell cycle (Eder, 1997). Each vial contains 1mg/ml of Cisplatin. Cisplatin has a molecular weight of 300.05

Etoposide

This topoisomerase II inhibitor, $C_{29}H_{32}O_{13}$, (VEPESID, Bristol-Myers Squibb Ltd.) appears to be effective in the G_2 phase of the cell cycle in mammalian cells (Gilman *et al*, 1996). Each vial contains 20mg/ml of Etoposide. Etoposide has a molecular weight of 588.58

Radiosensitiser

Pentoxifylline

This methylxanthine derivative, $C_{13}H_{18}N_4O_3$, was used at a concentration of 2mM, which is below the TD_{50} for all the cell lines used in the study. Each TRENTAL (Hoechst, Germany) infusion ampoule of 15ml contains 300mg of pentoxifylline. Pentoxifylline has a molecular weight of 278.3

Proliferation arrest of prostatic cells

4000 cells were seeded in 24-well multiwell plates with medium containing charcoal-stripped (cs) FBS (see Appendix). The medium for the DU 145 and BM1604 cell lines was supplemented with 10% csFBS, and 5% csFBS for the LNCaP cell line. 5-α dihydrotestosterone (DHT) was added a day later (2 days later in the case of LNCaP) in concentrations ranging from 0.001 – 100nM, for a period of 24 hours. The experiment was stopped after 4 days and the cell growth determined by crystal violet assay as described in Baker *et al* (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.

Growth curves and cell doubling times

Exponentially growing cells were trypsinised, counted and seeded at 2×10^4 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 (model Z_F). 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.

Drug toxicity

The ranges of drug concentrations for the various cell lines were determined as follows. Exponentially growing cells were trypsinised, counted and 4000 cells/ml/well seeded into 24-well multiwell plates. The cells were allowed to settle for 4-5 hours (for DU145 and BM1604) and 2 days (for LNCaP) before the addition of varying concentrations of a 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 and stained with 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.

Irradiation procedure

Cells were irradiated at room temperature (22°C) with ^{60}Co γ-irradiation. Two ^{60}Co γ-irradiation sources were used at Tygerberg Hospital, with a mean dose rate of 1.6105 Gy/min (1.19 - 2.03 Gy/min). 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 30-cm^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.

Clonogenic cell survival assay

Near-confluent stock cultures were washed with sterile 10% 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. Cells were allowed to settle for a minimum of 4 hours (2 days for LNCaP) before a dose of irradiation or pentoxifylline (at a final concentration of 2mM) was administered. The latter being added just prior to irradiation. The cells were exposed to the pentoxifylline for 24 hours and the medium replaced. Irradiation response curves were obtained by exposing cells to graded doses ranging from 0-10Gy. After an appropriate time of incubation (7-10 days) the colonies were fixed, stained and scored. The mean (± SD) of the surviving fractions for the three experiments were plotted against the irradiation dose. The quality of the fits is indicated by the correlation coefficient, R^2.

Radiosensitisation effect of pentoxifylline

Radiosensitisation induced by pentoxifylline (2mM) added immediately prior to irradiation, and pentoxifylline added at the time of maximum G_2 block expression was assessed by clonogenic

assay in the DU145, BM1604 and LNCaP cell lines. Cells were seeded in $25cm^2$ tissue culture flasks to yield approximately 100-200 colonies. After cell attachment and pentoxifylline treatment, the flasks were irradiated to 0, 2, 4, 6, 8, and 10Gy with ^{60}Co γ-irradiation. In samples where pentoxifylline was added at maximum G_2 block expression, the cells were exposed to a single dose of 7Gy ^{60}Co γ-irradiation. After an incubation period of ten days, colonies containing more than 50 cells were scored and counts adjusted for plating efficiency (PE). Radiosensitisation by pentoxifylline is expressed as an enhancement factor (REF), given by the ratio of survival factors, with and without pentoxifylline.

$$REF = \frac{SF\,(\text{irradiation})}{SF\,(\text{irradiation} + \text{pentoxifylline})}$$

Dose enhancement determination

At the time of the maximum G_2 block, 2mM pentoxifylline and a cytotoxic drug (at TD_{10}) were added to each flask for a period of 24 hours. In a separate set of flasks, the cytotoxic drug was added when the G_2 block was abrogated, and also exposed for 24 hours. The flasks were re-incubated for at least ten days after a medium change. Colonies were scored and the dose enhancement ratio (ER) was calculated thus,

$$EF_1 = \frac{SF\,(7Gy + \text{drug } TD_{10})}{SF\,(7Gy + PTX + \text{drug } TD_{10} \text{ at } G_2 \text{ block})}$$

$$EF_2 = \frac{SF\,(7Gy + drug\ TD_{10})}{SF\,(7Gy + PTX + drug\ TD_{10}\ when\ G_2\ block\ is\ abrogated)}$$

Flow Cytometry

Determination of DNA content

Analysis of DNA content was performed by flow cytometry as described elsewhere (Ormerod, 1990). Briefly, cells were trypsinised, pelleted by centrifugation at 600G for 5 minutes, vortexed in 200µl ice-cold PBS, fixed in 2ml 70% cold ethanol, and stored at –20°C for at least 24hrs. Cell pellets were then harvested by centrifugation and resuspended in 800µl PBS (to avoid clumping, cells were passed through a 26-gauge syringe needle). To this was added 100µl RNase (Sigma, South Africa) (1mg/ml) and 100µl PI (Sigma, South Africa) (400µg/ml) before incubation at 37°C for 30 minutes. Stained cells were kept at 4°C in the dark before analysis on a flow cytometer, FACScan© (Becton Dickinson).

For all procedures, cells were excited with a single 488nm argon laser and PI (red) fluorescence detected through a 600nm bandpass filter. Red fluorescence data (FL2-area and FL2-width) were collected in list mode format to 10 000 total events using the program, Cell Quest©. Cell cycle analysis was carried out using the program, ModFitLT© (gated to exclude cell debris, nuclei doublets and triplets).

Radiation induced G_2/M arrest, and abrogation by pentoxifylline

To establish the time of maximum G_2/M phase arrest, exponentially growing DU145, BM1604 and LNCaP cells were seeded in 25cm^2 flasks and exposed to a single ^{60}Co-γ dose of 7Gy. After irradiation, the cell cultures were trypsinised at different time intervals ranging from 10-48 hours,

fixed, stained with PI and subjected to flow cytometry (Ormerod, 1990).

Pentoxifylline, at a final concentration of 2mM, was added to a second set of flasks at the time of maximum G_2/M arrest. Samples from this set were harvested every 2 hours for a period of 24 hours to ascertain how long the G_2/M block took to abrogate. The percentages of G_1 and G_2 populations in control cells and pentoxifylline treated cells were estimated using LYSIS II software.

Immunochemical detection of $p21^{WAF1}$

$p21^{WAF1}$ detection performed was according to a modified protocol of Deptala *et al.* (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 X100 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 (PharMingen, clone 2G12, cat.no. 15441A), or 3µl of mouse anti-IgG1 (Dako, X0931) in a 1:30 titre as a negative 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, F-2012) for 1 hour in the dark at room temperature (see Appendix). Assessment of $p21^{WAF1}$ induction was done by flow cytometry measuring green FITC (FL1-H) versus red PI (FL3-H) on a FACScan.

Statistics

Data are presented as the mean ± standard deviation of three independent experiments as indicated by error bars (Figures) and each experiment was repeated three times. A two-sided t test was used to compare the means between two groups. A P-value of < 0.05 was considered statistically significant.

RESULTS

1. Drug toxicity data

The 50% toxicity doses (TD_{50}) for Vinblastine, Cisplatin and Etoposide, established by crystal violet assay, are summarised in Table 1. It is apparent that the mutant cell lines, DU145 and BM1604, are more drug sensitive than the wild-type cell line, LNCaP. Vinblastine emerged as the most toxic drug with TD_{50} values ranging from 2.93nM - 4.11nM. Cisplatin takes an intermediate position with TD_{50} values ranging from 0.77μM - 8.20μM. Etoposide emerged as the least toxic drug with TD_{50} values ranging from 0.80μM - 9.50μM.

Table 1: TD_{50} drug concentrations for 3 human prostate carcinoma cell lines.

CELL LINE	VINBLASTINE	CISPLATIN	ETOPOSIDE
DU145 (TP53 mutant)	3.46nM	1.15μM	0.80μM
BM1604 (TP53 mutant)	2.93nM	0.77μM	0.82μM
LNCaP (TP53 wild-type)	4.11nM	8.20μM	9.50μM

Cell survival curves in Figures 3 and 4 serve to derive the 10% toxic dose (TD_{10}). These values are listed in Table 2 and were used to study the influence of G_2 block abrogation on drug toxicity. The normal clinical drug doses and the TD_{10} values are compared in Table 3, indicating that the doses employed in the clinic are 2-3 orders of magnitude higher.

Table 2: TD_{10} drug concentrations for 3 human prostate carcinoma cell lines.

CELL LINE	VINBLASTINE	CISPLATIN	ETOPOSIDE
DU145 (TP53 mutant)	1.26nM	0.31μM	0.15μM
BM1604 (TP53 mutant)	0.77nM	0.16μM	0.19μM
LNCaP (TP53 wild-type)	0.51nM	1.20μM	1.30μM

Table 3: Standard therapeutic dose range of cytotoxic drugs in nM and μM compared with the effective *in vitro* dose at the G_2/M Block.

Cytotoxic agent	TD_{10} values at G_2/M Block	Clinical dose
Vinblastine	0.51-1.26nM	2300-8450nM
Etoposide	0.15-1.30μM	33-94μM
Cisplatin	0.16-1.20μM	148μM

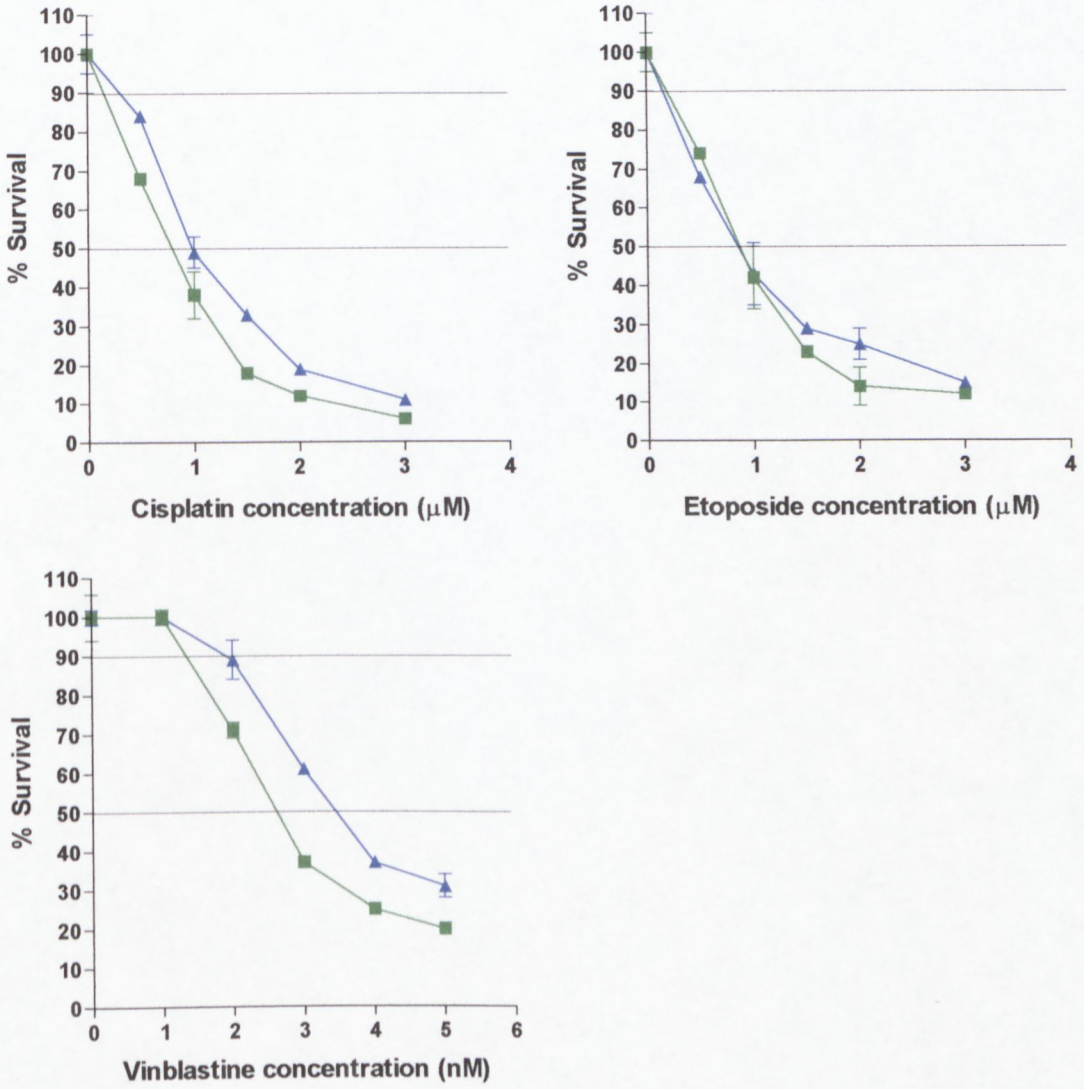

Figure 3: Dose response curves of Cisplatin, Etoposide and Vinblastine for the 2 human prostate cell lines BM1604 (■) and DU145 (▲).

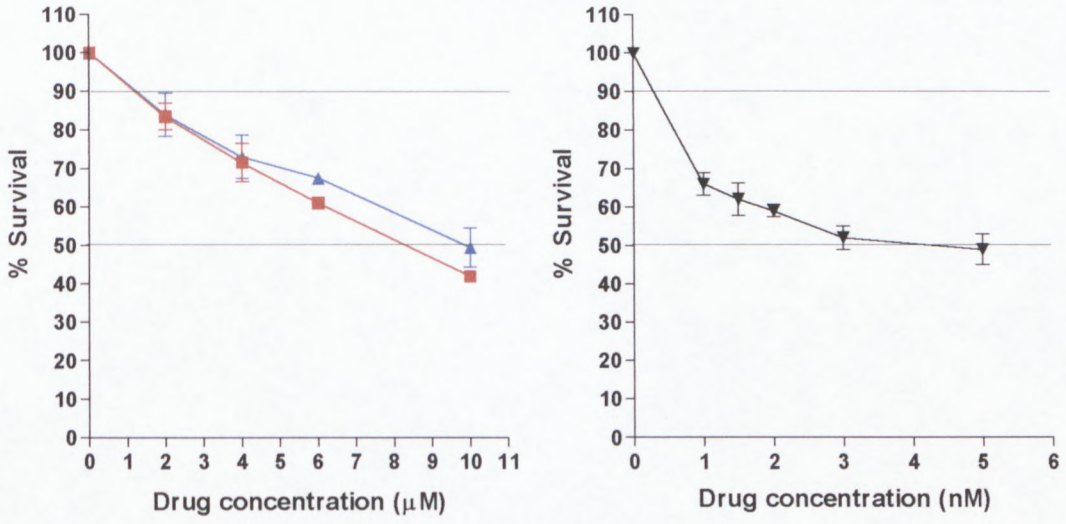

Figure 4: Dose response curves of Cisplatin (■), Etoposide (▲) and Vinblastine (▼) for the human prostate cell line LNCaP.

2. Pentoxifylline toxicity

The TD_{50} of pentoxifylline (toxic dose resulting in a cell kill of 50%) was found to be 4.25, 4.30, and 4.28mM for DU145, BM1604 and LNCaP cells, respectively (data not shown). A sub-toxic dose of 2mM pentoxifylline was used for all subsequent experiments.

3. Clonogenic survival assay

In the colony assay, the TP53 mutant prostate cell line, DU145, emerged as the most radiation resistant cell line, showing an SF_2 of 0.60. The TP53 mutant cell line, BM1604, held an intermediate position with an SF_2 of 0.47, while the TP53 wild-type cell line, LNCaP, emerged as the most radiation sensitive, showing an SF_2 of 0.28 (Table 4, Figure 5).

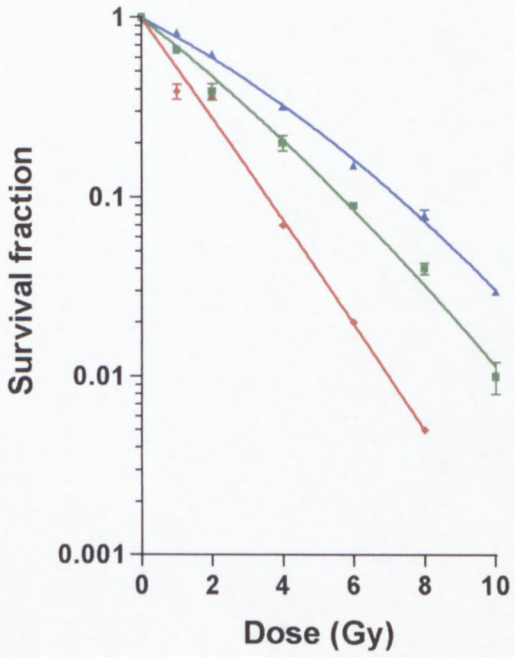

Figure 5: Cell survival of human prostate cell lines, BM1604 (■), DU145 (▲) and LNCaP (◆), following exposure to ^{60}Co γ-irradiation.

Table 4: Cell survival parameters for three human prostate cell lines following exposure to ^{60}Co γ-irradiation.

Cell line	α	β	α/β	SF_2	R^2
DU145	0.233	0.012	19.4	0.60	0.9981
BM1604	0.355	0.009	39.4	0.47	0.9933
LNCaP	0.632	0.0037	170.8	0.28	0.9919

4. Effect of pentoxifylline on radiotoxicity

When pentoxifylline was given just prior to irradiation it was found to exert a radiosensitising effect

particularly in TP53 mutant cells (Figure 6, Table 5). The radiosensitivity enhancement factors (REF) for three prostate cell lines are given in Table 6. The cell line, BM1604, showed REFs of 3.5, 6.67 and 16.7, for the 6, 8, and 10Gy dose points respectively, compared to a REF of 0.97 when pentoxifylline was added at the G_2 block maximum.

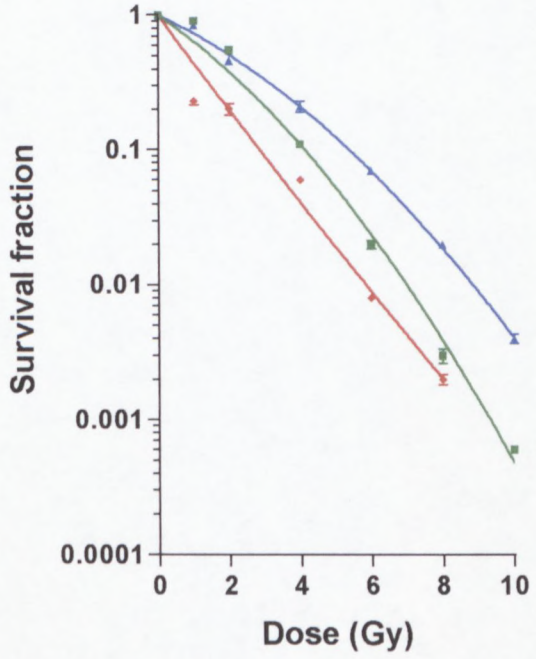

Figure 6: Cell survival of three human prostate cell lines, BM1604 (■), DU145 (▲) and LNCaP (◆), following exposure to ^{60}Co γ-irradiation and pentoxifylline added just prior to irradiation.

Table 5: Cell survival parameters for three human prostate cell lines following exposure to ^{60}Co γ-irradiation and pentoxifylline at 0hrs.

Cell line	α	β	α/β	SF_2	R^2
DU145	0.28	0.027	10.4	0.512	0.9987
BM1604	0.41	0.036	11.4	0.39	0.9923
LNCaP	0.82	0.005	164.0	0.199	0.9782

Table 6: Radiation toxicity enhancement factors (REFs) induced by pentoxifylline in various prostate tumour cell lines. REF is defined as the survival ratio of control cells in relation to survival in the presence of pentoxifylline. Pentoxifylline was added before irradiation. SF = survival fraction of the cells based on the colony assay.

	CELL LINES		
SURVIVAL FRACTION	**BM1604**	**DU145**	**LNCaP**
SF1	1.0	1.08	0.77
SF2	1.27	1.43	1.0
SF4	2.59	1.76	0.80
SF6	3.5	2.29	0.90
SF8	6.67	3.0	0.85
SF10	16.7	7.5	0.83

Footnote: When pentoxifylline was added at maximum expression of the G_2 block the corresponding radiotoxicity enhancement factors, REFs, at SF7 were found to be 0.97, 1.03 and 1.1 for BM1604, DU145 and LNCaP cells, respectively.

When pentoxifylline was present at the time of irradiation in DU145 cells, REFs at 6, 8, and 10Gy were found to be 2.29, 3.0 and 7.5 respectively, as compared to 1.03 when pentoxifylline was added at the G_2 block maximum.

In the wild-type cell line, LNCaP, no radiosensitisation effect was detected when pentoxifylline was added either before irradiation, or at the G_2 block maximum.

5. Influence of irradiation on cell cycle progression

Exposure of prostate cell lines to 7Gy ^{60}Co γ-irradiation induced cell cycle delays at the G_1 and G_2 cell cycle phases.

In BM1604 and DU145 cells, which are TP53 mutant, the cells responded to ^{60}Co γ-irradiation damage by arresting in the G_2/M phase. In the LNCaP, TP53 wild-type cell line, a pronounced G_1 phase delay, and a smaller G_2/M phase delay was observed (Figure 7).

In the TP53 mutant cell lines, DU145 and BM1604, the G_2/M delay was maximally expressed 12 and 18 hours after irradiation, respectively. The normal recovery time from the cell cycle block was approximately 54 and 66 hours after irradiation, respectively.

In the TP53 wild-type cell line, LNCaP, the time of maximum expression of G_1 and G_2 blocks occurred after 42 hours, with a normal recovery time of approximately 100 hours.

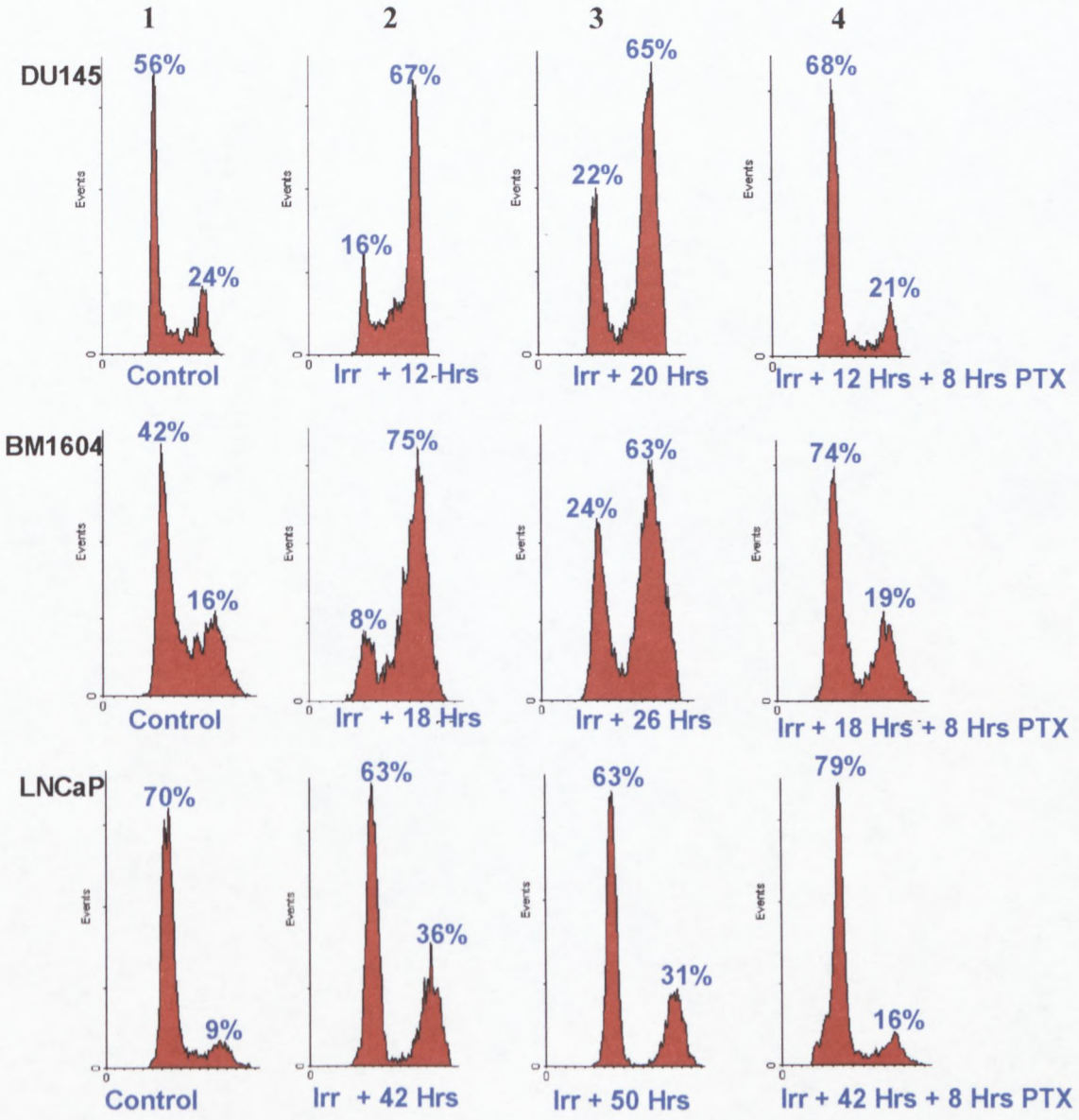

Figure 7: DNA histograms showing the influence of 7Gy of irradiation, and 7Gy of irradiation plus pentoxifylline added at maximum G_2 block, on the distribution of cells in G_1 and G_2 cell cycle phases at various post-irradiation times in TP53 mutant, DU145 and BM1604 cells, and TP53 wild-type LNCaP cells.

Comparison of column 2 to 3 shows normal cell cycle recovery.

Comparison of columns 2 and 4 shows the influence of 2mM pentoxifylline exposure for 8 hours.

It is apparent that pentoxifylline effectively accelerates cell cycle recovery and, hence, abrogates the

cell cycle block at G_2/M.

6. Influence of hormone status

Crystal violet vital dye staining assays showed that the addition of DHT was of no consequence to cell growth in the TP53 mutant cell lines, BM1604 and DU145 (Figure 8). However, when the TP53 wild-type cell line, LNCaP, was subjected to charcoal-stripped medium and DHT was then added at concentrations of 0.001 – 1.0nM, growth was restored to a level of 50% of that of the control. 1nM seems to be saturating because increase of the DHT to 100nM shows no further growth stimulation. The fact that growth of LNCaP cells was not restored to the control level by DHT indicates that essential factors other than steroids, removed by charcoal treatment, were important for optimal growth. In charcoal-stripped media (5% csFBS) the proliferation rate of LNCaP cells declined to 28% as compared to that in normal media (5% FBS).

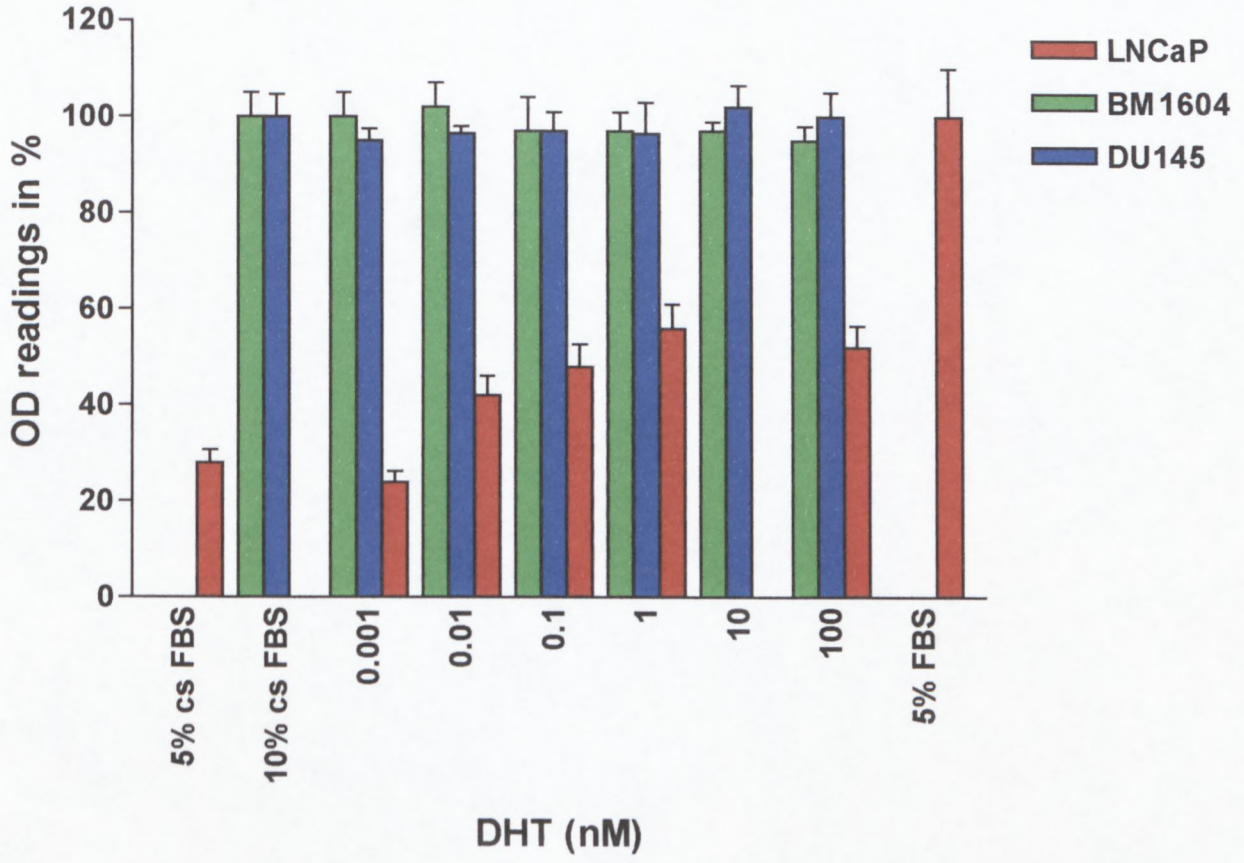

Figure 8: The effect of DHT addition to charcoal-stripped medium on the proliferation of 3 prostate carcinoma cell lines.

7. Induction of p21

The TP53-dependent induction of the $p21^{WAF1}$ gene was used to assess the functionality of the TP53 gene. It was found that the TP53 wild-type cell line, LNCaP, expressed functional TP53 as indicated by the induction of the p21 target gene (Figure 9). In the two mutant cell lines, DU145 and BM1604, no baseline p21 expression could be detected. In LNCaP cells no p21 induction was apparent during the initial 2 hours post-irradiation but p21 induction reached a peak 4 hours post-irradiation (7Gy ^{60}Co γ-irradiation). This level had not decreased appreciably 20 hours later (24

hours post-irradiation). This indicates that TP53 was functional.

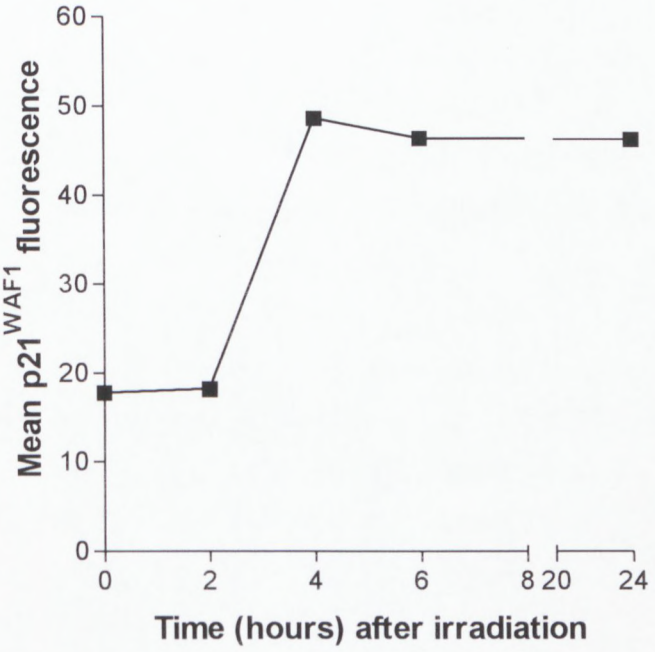

Figure 9: The response of TP53 wild-type LNCaP cells to 7Gy ^{60}Co γ-irradiation in terms of nuclear accumulation of p21^{WAF1}

8. Influence of G_2 block abrogation on the toxicity of Cisplatin, Etoposide and Vinblastine

Prostate cells were irradiated with 7Gy ^{60}Co γ-irradiation, stained with PI, and DNA content was monitored by flow cytometry. At maximum expression of the G_2 block, pentoxifylline and a TD_{10} dose of the anticancer drug was added, and cell survival determined, using the survival of 7Gy + drug alone as controls. It was found that addition of the drug at G_2 maximum, and 8 hours later, markedly enhanced the toxicity (Figure 10).

The toxicity enhancement factors (EFs) tended to be greater in the TP53 mutant cell lines, DU145 and BM1604, than in the TP53 wild-type cell line. The drug response of the TP53 wild-type cell

line, LNCaP, to irradiation and drugs under abrogating conditions was weak for all three drugs, showing EFs of 1.10, 1.21, and 1.47 for Etoposide, Vinblastine and Cisplatin, respectively, when the drugs were administered at the G_2 block maximum. When the drugs were administered 8 hours after the G_2 block maximum, EFs were found to be 1.50, 1.38 and 1.57 for Etoposide, Vinblastine and Cisplatin, respectively (Tables 7 and 8).

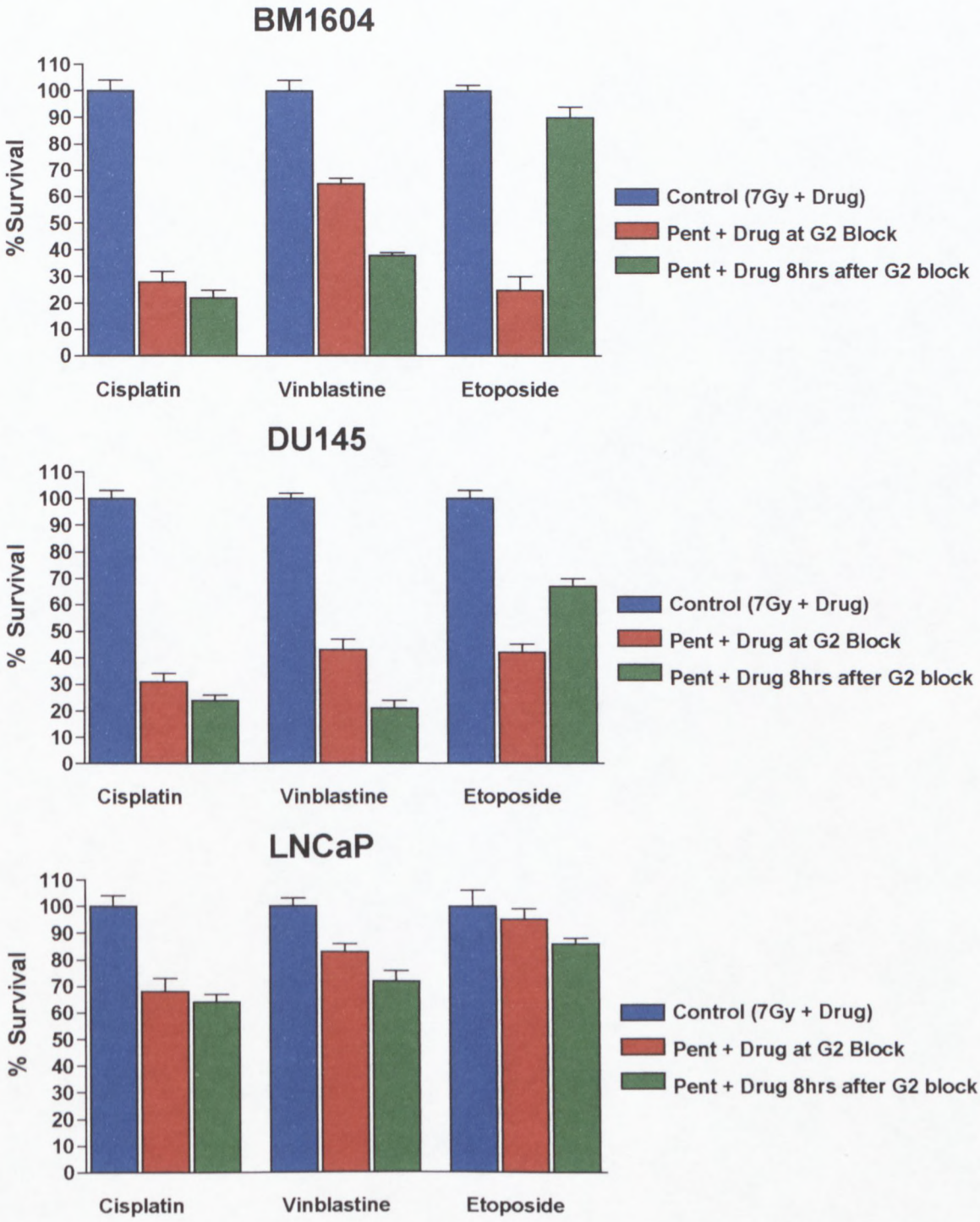

Figure 10: Influence on cell survival of Cisplatin, Vinblastine and Etoposide in TP53 mutant (BM1604 and DU145) and TP53 wild-type (LNCaP) prostate cancer cell lines under conditions of G_2 block abrogation. Survival at 7Gy and drug alone served as the control. Drug was added at a toxic dose of 10% (TD_{10}). Pentoxifylline was added at maximum G_2 block expression.

Table 7: Dose enhancement factors (EFs) for Cisplatin, Vinblastine and Etoposide, added at maximum expression of the G_2/M block.

Treatment	DU145	BM1604	LNCaP
Irrad + 2mM Ptx + Cisplatin at TD_{10}	3.30	3.60	1.47
Irrad + 2mM Ptx + Vinblastine at TD_{10}	2.30	1.53	1.21
Irrad + 2mM Ptx + Etoposide at TD_{10}	2.40	4.00	1.10

Table 8: Dose enhancement factors (EFs) for Cisplatin, Vinblastine and Etoposide, added 8 hours after maximum expression of the G_2/M block.

Treatment	DU145	BM1604	LNCaP
Irrad + 2mM Ptx + Cisplatin at TD_{10}	4.11	4.50	1.57
Irrad + 2mM Ptx + Vinblastine at TD_{10}	4.82	2.60	1.38
Irrad + 2mM Ptx + Etoposide at TD_{10}	1.50	1.00	1.50

In the mutant cell lines, DU145 and BM1604, enhancement factors (EFs) were 3.30 and 3.60 for Cisplatin, respectively, 2.30 and 1.53 for Vinblastine, respectively, and 2.40 and 4.00 respectively, for Etoposide, when the drug was added at maximum G_2 block expression (Table 7).

Enhancement factors (EFs) for DU145 and BM1604 were 4.11 and 4.50 respectively, for Cisplatin, 4.82 and 2.60 respectively, for Vinblastine, and 1.50 and 1.00 respectively, for Etoposide, when the drug was added 8 hours after maximum G_2 block expression (Table 8).

DISCUSSION

I have studied the influence of G_2 block abrogation on drug toxicity in TP53 wild-type, LNCaP, and TP53 mutant, DU145 and BM1604, prostate cell lines. Pentoxifylline was used as the abrogating drug at a concentration of 2mM, which is well below the TD_{50} of 4.3mM. Other studies have also employed a 2mM concentration (Russell *et al*, 1996). The response to chemotherapeutic agents was examined using the microtubule inhibitor, Vinblastine, the topoisomerase II inhibitor, Etoposide, and the DNA cross-linking drug, Cisplatin. The effectiveness of pentoxifylline to abrogate the G_2 block and promote mitosis was confirmed by flow cytometry (Russell *et al*, 1995). In agreement with other reports a clear G_2 block is only seen in TP53 mutant cells (Hollstein *et al*, 1991). TP53 wild-type cells show a G_1 block after irradiation and an elevated G_2 population which only declines when the G_1 block resolves (Figure 7, column 4). In these cell lines abrogation of a fully established G_2 block, induced by a dose of 7Gy, takes approximately 8 hours (Figure 7) as compared to 50-100 hours in controls not receiving pentoxifylline.

The three cell lines used required the same time to undergo complete G_2 block abrogation, irrespective of their normal G_2 block relaxation times. Pentoxifylline resolves the G_2 block of TP53 wild-type and mutant cells by inducing mitosis. This also precipitates a G_1 overshoot, and cells resume DNA synthesis 6-8 hours after G_2 block maximum (Figure 6). The G_1 overshoot probably arises from a cohort of G_2 cells moving into G_1. This phenomenon is seen in TP53 wild-type and TP53 mutant cells after G_2 block abrogation and is consistent with previous observations (Russell *et al*, 1996). The G_1 overshoot can be interpreted as a TP53-independent, temporary second G1 block with unknown function (Binder *et al*, 2000). In view of the preferential sensitising effect of pentoxifylline for TP53 mutant cells to DNA damage, the influence of Etoposide, Vinblastine and Cisplatin was investigated at two points: (1) when cells recover normally from a G_2/M block, and

(2) when the process is abrogated by pentoxifylline.

The drugs in question were administered at essentially non-toxic concentrations (TD_{10}) under abrogating conditions (together with 2mM pentoxifylline). Tables 7 and 8 show that the cytotoxicity of the three common cancer drugs, Etoposide, Cisplatin and Vinblastine, is markedly enhanced in the TP53 mutant cell lines, DU145 and BM1604. The fact that G_2 block abrogation produces larger enhancement factors (EFs) in TP53 mutant cells but not in TP53 wild-type cells, is in line with results on TP53 mutant squamous cell carcinoma (SCC) and melanoma cells. In these cell lines Cisplatin, Melphalan and Daunorubicin were observed to be up to 80X more effective when given under conditions of G_2 block abrogation (Binder *et al*, 2000). The results on prostate cell lines and the three drugs, Cisplatin, Vinblastine and Etoposide, also affirm that early entry into mitosis is a critical event which renders these cells particularly drug sensitive. In TP53 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 enters the G_1 phase and toxicity enhancements are reduced.

Etoposide has been shown to operate in the G_2 phase of the cell cycle (Gilman *et al*, 1996). In our experiments, toxicity enhancements (Table 7) were indeed found to be higher at this point in the cell cycle. The action of Cisplatin is independent of cell cycle phases (Eder, 1997) and the toxicity is high in the TP53 mutant cell lines, particularly in the post-G_2 phase. (Table 8). Vinblastine prevents microtubule assembly causing cells to arrest in the late G_2/M phase by preventing formation of mitotic filaments for nuclear and cell division (Eder, 1997). Therefore, a marked toxicity enhancement would be expected under conditions of G_2 block abrogation (Table 8).

Drug resistance from P-glycoprotein induction is very frequent in TP53 mutant tumours. Since P-glycoprotein plays an important role in drug detoxification (Higgins *et al*, 1997), it can be expected that enhancement factors achieved by G_2 block abrogation will vary between cell lines and tumour

types (Ribeiro *et al*, 1997). P-glycoprotein induction was not determined here.

No radiosensitisation effect was detected when pentoxifylline was added at the G_2 block maximum (Table 6). Irrespective of TP53 status, REFs were found to be in the region of 1.0 for all three cell lines. When pentoxifylline is added immediately before or after irradiation, REFs are known to be in the region of 1.2-2.0. This is in agreement with other reports on caffeine and pentoxifylline (Theron and Böhm, 1998). At 2Gy the TP53 mutant prostate cell lines, DU145 and BM1604, show REFs of 1.43 and 1.27 respectively (Table 6). In V79 and HeLa cells REFs at 2Gy have been found to be 1.4 – 1.6 (Vernimmen *et al*, 1994). Results on human colon and cervical cancers show REFs of 1.23 – 1.99 (Li *et al*, 1998).

From the very low REFs shown by pentoxifylline alone at the G_2 maximum and the high REFs shown when pentoxifylline is added immediately before irradiation, it can be concluded that the mechanisms of action are not the same. Early addition of the methylxanthine has generally been linked with repair inhibition, and the reduction of recovery ratios supports this view (Vernimmen *et al*, 1994). Residual unrepaired double-strand breaks (dsb) remaining after high dose irradiation strongly suggest that repair inhibition is one mechanism by which pentoxifylline sensitises cells to irradiation (Theron and Böhm, 2000). Late addition of pentoxifylline at the G_2 maximum, on the other hand, seems to operate mainly on the cell cycle where the drug functions as a G_2 block abrogator (Russell *et al*, 1996). That this scenario is not toxic to cells could be due to the fact that repair takes place within 2-12 hours, and before the G_2 block is fully expressed. This distinction must be noted when assessing the potential of methylxanthines as adjuvants to irradiation or chemotherapy.

LNCaP is a well-differentiated human prostatic cancer cell line, and contains a mutated, but functional, androgen receptor. This would allow the cells to respond to androgen stimulation and to

proliferate (Horoszewicz *et al*, 1983). It is shown here that prostate cells responded differently to androgen, also showing saturation kinetics. At 10^{-12} – 10^{-9}M concentrations of DHT cells undergo active proliferation. At a 10^{-7}M concentration of DHT cells entered growth arrest (Langeler *et al*, 1993; Lee, 1997). The role of androgen in prostatic epithelial cell proliferation, however, is still not fully understood. In cultures of benign prostatic epithelial cells, androgen is not considered a direct mitogen (Lee *et al*, 1995). DHT has a dose-related mitogenic effect on LNCaP cells only if its concentration in the medium is less than 10^{-9}M. DHT concentrations higher than 10^{-9}M cause a dose-related inhibition of LNCaP proliferation.

When pentoxifylline is used as an irradiation modifier SF6-SF10 alone show that BM1604 is more sensitised than DU145 by a factor of 1.5-2.2 (Table 6). When pentoxifylline is used as a drug modifier at G_2 maximum, BM1604 and DU145 undergo similar enhancements by factors of 2-4. LNCaP factors are 1.10-1.60 (Tables 7 and 8). This suggests that the modification of radiosensitivity by pentoxifylline alone and the drug toxicity enhancements induced by G_2 block abrogation affirm that the TP53 status and cell type play a role in the potential of cells to respond to these modifications.

The chemotoxicity of prostate tumour cell lines *in vitro* can, as shown here, be enhanced by the application of a low dose of drug with a G_2 block abrogator. TP53 mutant and androgen independent cells, particularly, were sensitised to a subsequent toxic intervention. The time dependence of the process, as seen here, may rest solely on the mode of action of the chemotherapeutic drugs employed. The natural history of prostate cancer offers several occasions for early application of chemotherapy. The administration of drugs in combination with irradiation at a time when the tumour burden is low, could make tumour control more effective and accomplish better survival.

CONCLUSIONS

Drugs which influence cell cycle checkpoint control have attracted considerable interest in tumour therapy. It is shown that pentoxifylline at subtoxic concentrations effectively abrogates the G_2 block in TP53 mutant prostate cell lines and that these conditions are favourable to accomplish marked enhancements of the toxicity of anti-tumour drugs. Pentoxifylline concentrations, which are tolerated in humans, are 10-30μM (Russell *et al*, 1996). This concentration is still below the threshold for radiosensitisation. The search for new compounds which are effective at inhibiting the G_2 checkpoint at low systemic toxicity continues (Bunch and Eastman, 1996). Staurosporine analogues have been shown to abrogate G_2 blocks at nM concentrations and appear to be well tolerated (O'Connor, 1996). The methylxanthine derivative, lisofylline, also abrogates G_2 blocks and is better tolerated than pentoxifylline (Böhm *et al*, 2000).

Checkpoint-based strategy to sensitise TP53 mutant prostate tumours is clinically still unexplored and clearly deserves attention.

APPENDIX 1

Fixative for colony assay

1 part Methanol
1 part Glacial acetic acid
8 parts deionised water

Staining solution for colony assay

0.01% Amido black dissolved in the abovementioned fixative

5α-Dihydrotestosterone

Stock solution (10^{-3}M) prepared by dissolving the 5α-dihydrotestosterone ($C_{19}H_{30}O_2$) (Sigma, South Africa) in absolute ethanol.
Sterile filtered (0.22μm) working solutions ranged from 0.001nM to 100nM.

Buffered formalin fixative for 24-well multiwell plate assay

4g KH_2PO_4
4.45g $Na_2HPO_4.2H_2O$
Dissolve the above ingredients in deionised water, and add 100ml formalin.

Staining solution for 24-well multiwell plate assay

0.01% Crystal violet dissolved in deionised water.

Sodium Dodecyl Sulphate (SDS)

A 10% solution of SDS (SaARchem, South Africa) in deionised water was used to dissolve the stain in the crystal violet assay.

Phosphate Buffered Saline (PBS)

8g NaCl
1.43g $Na_2H(PO_4).2H_20$
0.2g KCl
0.2g KH_2P0_4
Dissolve the ingredients, make up to 1L with deionised water, pH to 7.3, and sterile filter with a 0.22μm Millipore (Massachusetts, USA) filter unit.

Trypsin

8g NaCl
0.4g KCl
1g Glucose
0.58g $NaHCO_3$
0.5g Trypsin 1 : 250
Dissolve the above ingredients, make up to 1L with deionised water, sterile filter with a 0.22um filter unit, and store at –20°C as a 0.05% trypsin solution.

1% PFA

1g PFA
50ml deionised water
2 drops 10N NaOH or
20 drops 1N NaOH
Heat for 30 min at 60°C. Prepare fresh.

1% BSA, in PBS

1g BSA
99g PBS
Prepare fresh.

Antibody p21

p21 2G12	0.5µg ~ 1µl
1% BSA/PBS	100µl

Use 100µl per sample

Isotype control

IgG1	0.1µg ~ 3µl
1% BSA/PBS	100µl

Use 100µl per sample

Anti-mouse IgG antibody

FITC goat anti-mouse	3µg ~ 3µl
1% BSA/PBS	100µl

Use 100µl per sample.

PI / RNAse A

PI in PBS (1mg/ml)	5µl	
RNAse A (1mg/ml)	50µl	freshly prepared
PBS (+ Mg/Ca)	445µl	

Use 500µl per sample.

APPENDIX 2

Protocol for p21 protein detection

Fixation

Fix 1-2 x 10^6 cells in 1 ml of methanol free, freshly prepared 1% formaldehyde in PBS.

Leave for 15 minutes at 4°C.

Add 5 ml of PBS and centrifuge for 5 minutes at 300G.

Re-suspend the cell pellet in 200 µl of PBS and add 2 ml of ice cold 80% Ethanol (-20°C).

Leave at least 10 minutes (can be stored at -20°C for weeks).

First antibody

Add 5 ml of PBS at room temperature for 10 minutes, then centrifuge (300G for 5 min).

Add 1 ml of 0.25% Triton X-100 / PBS, leave on ice block in styrofoam box for 5 minutes.

Add 1 ml 1% BSA / PBS, centrifuge (300G for 5 min).

Suspend the cell pellet in 100µl 1% BSA / PBS containing the 1. Ab p21 (PharMingen, clone 2G12, cat.no. 15441A) in 1:100 titre (1µl per sample ~ 0.5 µg, stock is 0.5 mg/ml, quantity 200 µ l).

Prepare a negative isotype control: 100µl 1% BSA / PBS containing isotype IgG1 (Dako, X0931) in 1:30 titre (3 µl per sample ~ 0.1µg)

Incubate at 4°C overnight in the dark on a shaker (alternatively, for 3hrs at room temperature).

Second antibody

Add 1 ml 1% BSA / PBS and centrifuge.

Take up the pellet in 100µl of 1% BSA / PBS containing fluorescein-conjugated goat anti-mouse IgG Ab (Sigma F-2012, anti-mouse IgG, whole molecule) in 1:30 titre (3µl per sample ~ 3µg).

Incubate for 60 minutes in the dark on a shaker.

Add 1 ml 1% BSA / PBS and centrifuge.

Re-suspend the cell pellet in 500µl solution containing 10µg/ml PI (Sigma, P-4170), 100µg/ml RNAse A (Sigma, R-5125), (stock PI : 1 mg/ml in PBS; stock RNAse A fresh 1mg/ml in PBS).

Incubate for 30 minutes in the dark at 37°C.

Survival curves

A survival curve describes the relationship between radiation dose and the probability of cells to survive a given level of radiation dose absorbed. The capability of a single cell to proliferate into a large colony or clone of at least fifty cells is proof that it has retained its reproductive capacity. Examples of survival curves are given in Figure 4. These curves are plotted on a logarithmic scale for the following reasons:

1) If cell death is a random event then the survival rate will be an exponential function of dose and this will be a straight line on a semi-log plot.
2) A logarithmic scale allows us to view the effects of radiation over a wide range of doses with more ease.

Cell survival curves can be described by the linear-quadratic equation:

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

where S is the proportion of cells surviving at a dose D, α is the coefficient associated with lethal damage and the β parameter is related to repairable damage (Fertil *et al*, 1984). As the linear-quadratic model is most suitable to describe survival curves it was used in this study.

Clonogenic cell survival has long been considered the standard for cellular radiation response. The formation of a microscopic colony from a single cell requires sustained cell division and as such is the ultimate proof of reproductive integrity. A number of non-clonogenic assays have also been developed based on cell growth in a multiwell plate. This includes the crystal violet assay used in this study, as described in Mitchell (1988). A colorimetric assay enables one to calculate cell survival parameters and assess the radiosensitivity of cell types that do not form colonies. It is an endpoint somewhat different from clonogenic assays and has certain limitations. The technique has the advantage that it is much simpler and faster than the clonogenic assay and that it can be automated. This method, however, generally gives an over-estimation of the survival rate compared to the clonogenic assay, since it measures both dividing and non-dividing cells.

Preparation of charcoal-stripped foetal bovine serum

100ml FBS + 10g activated charcoal (Sigma, South Africa) + 1g dextran 70kD (Sigma, South Africa).

Mix, and leave at 4°C for 24hrs.

Centrifuge at 10 000G for 1hr at 4°C.

Centrifuge the supernatant at 27 000G for 30 minutes.

Sterile filter (0.22μ) the supernatant.

A control sample consisting of 2ml FBS + 8ml deionised water + tritiated 5-α dihydrotestosterone was treated, as in the method above, and the level of radioactivity checked, before and after charcoal stripping, on a Beckman LS 5000TD Liquid Scintillation Counter (USA), before declaring the product ready for use.

Flow cytometry

A short description of the basic principles of flow cytometry is described here as it was used in this study to analyse DNA content. This technique is used for making rapid measurements of particles or cells as they flow one by one in a fluid stream through a sensing point. Determinations of cell numbers, cell size, cell shape and a variety of other cellular characteristics such as DNA content, surface antigens, enzyme activity, calcium flux and pH can be made. The latter require the incorporation or attachment of fluorescent dyes which are excited by a laser. The scattered and fluorescent light generated by cells passing through the illuminating beam is collected by photodetectors which convert the photon pulses into electronic signals. Further electronic and computational processing results in the graphic display and statistical analysis of the measurements being made. A generalised flow cytometry system is shown in Figure 11.

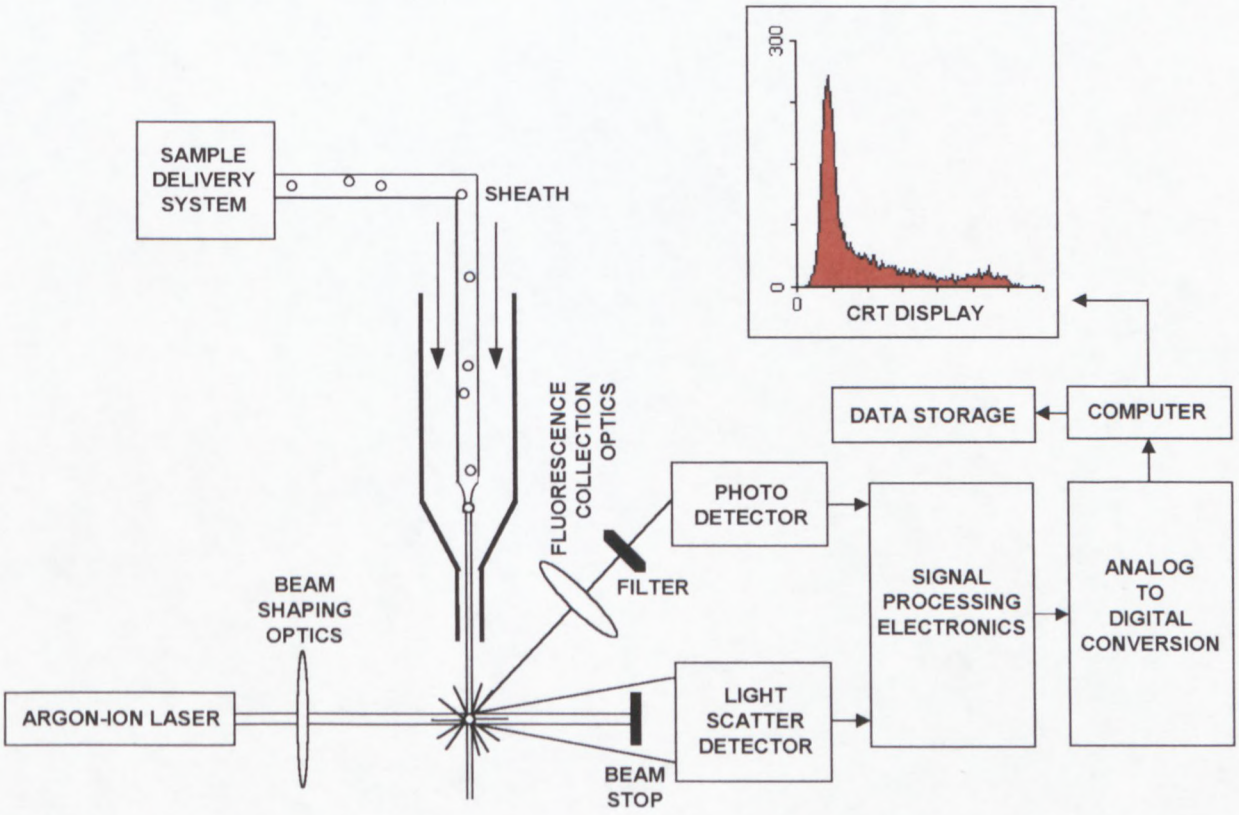

Figure 11: A generalised flow cytometry system (Wheeless, Jr., 1991)

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