Urol Res (2001) 29: 221–227

ORIGINAL PAPER

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Chemosensitivity of prostatic tumour cell lines under conditions of G_2 block abrogation

Received: 23 November 2000 / Accepted: 23 February 2001

Abstract Conventional chemotherapy has had very limited success in the control of hormone-refractory prostate cancer. Methylxanthine derivatives, such as pentoxifylline (PTX), are known to abrogate the G_2 block and enhance the toxicity of ionising irradiation and chemotherapeutic agents. It is now also established that late addition of the cytotoxic drug after irradiation under conditions of G_2 block abrogation sensitises human tumour cells for cytotoxins. Here we assess whether the chemosensitivity of prostate tumour cell lines can be enhanced by the application of a low dose of drug in conjunction with a G_2 block abrogator. Prostate cell lines DU145, BM1604 and LNCaP were irradiated with 7 Gy ^{60}Co γ-irradiation. A sub-toxic (2 mM) dose of pentoxifylline and a cytotoxic drug were added at maximum expression of the G_2 cell cycle block and cell survival was determined by colony assay. Cisplatin, etoposide and vinblastine were tested at a toxic dose of 10% (TD_{10}). In the TP53 mutant cell lines, DU145 and BM1604, dose enhancement factors (EFs) were found to be in the region of 4.20 for cisplatin, 3.70 for vinblastine, and 3.20 for etoposide. In the TP53 wild-type cell line, LNCaP, the enhancement factors were low and in the region of 1.20 for cisplatin, vinblastine and etoposide. It is clear, therefore, that toxicity enhancement factors (EFs) are greater in the TP53 mutant cell lines, DU145 and BM1604, than in the TP53 wild-type cell line, LNCaP. The results indicate that a significant enhancement of drug toxicity can be obtained if the cytotoxic drug is given under conditions of G_2 block abrogation. The sensitisation of prostate cancer cells to cytotoxic drugs is particularly high in radiation-resistant TP53 mutant tumour cells. Drugs which abrogate G_2 block have the potential to enhance the therapeutic index and therefore reduce the toxicity of chemotherapy drugs.

Key words G_2/M block abrogation · Cytotoxicity · Prostate cell lines · Pentoxifylline

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Introduction

Mortality and morbidity in prostate cancer commonly arise in metastatic disease, particularly in hormone-refractory disease [15]. At present, chemotherapy, other than hormonal ablation, has little or no therapeutic benefit [12]. In the poor outcome of chemotherapy it must be noted that drugs are commonly given when the tumour burden is high [6], and the response of normal rapidly proliferating cells is dose limiting [17]. The high drug toxicity therefore operates against normal tissue tolerance [5]. The development of new agents with specific cellular targets and knowledge of innate damage response mechanisms of tumour cells offers new strategies for therapy. Genotoxins activate cell cycle checkpoints at the G_1/S and G_2/M transitions of the cell cycle. The resultant cell cycle blocks are thought to prevent the replication and propagation of defective genomes. In TP53 mutant cells where the G_1/S checkpoint is absent, DNA damage inflicts a G_2 block from which cells recover after 40–60 h and then re-enter the cell cycle [2]. We have shown that abrogation of the G_2 checkpoint is an effective strategy whereby TP53-defective cells can be targeted for destruction by a second irradiation or chemotherapeutic insult [2]. The complete molecular mechanism of G_2 block abrogation by pentoxifylline has not yet been elucidated but cyclin B1 and $p34^{CDC2}$ levels are rapidly restored after irradiation and facilitate early entry of G_2 cells into mitosis [18, 19]. However, early entry into mitosis alone is not lethal and presence of a cytotoxic drug is required to achieve enhanced cell inactivation [20]. In the following study, we have assessed the influence of G_2 block abrogation on

chemosensitivity in prostate tumour cell lines of defined TP53 and androgen status. The enhancement of drug toxicity was significantly greater ($P < 0.05$) when the drug (cisplatin and vinblastine) was added 8 h after G_2 block abrogation, as opposed to drug addition at maximum G_2 block expression.

Materials and methods

Cell culture

The TP53-mutant prostate cell lines, DU145 and BM1604, were obtained from Highveld Biological, South Africa. The TP53 wild-type cell line, LNCaP.FGC, was obtained from Dr. J.H. Visser at the University of Stellenbosch Medical Faculty (Department of Urology). DU145 cells were grown in minimum essential medium (MEM; Sigma) supplemented with 10% heat-inactivated foetal bovine serum (FBS). BM1604 and LNCaP cells were grown in RPMI-1640 medium supplemented with 10% and 5% FBS, respectively. All the media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Cell cultures were maintained at 37 °C with 5% CO_2 in 75 cm^2 cell culture flasks (Falcon Plastics). All cell culture consumables were obtained from Highveld Biological, unless stated otherwise. LNCaP cells were left undisturbed for 48 h to facilitate attachment, as previously described [10].

Drug and chemical preparation

The cross-linking agent cisplatin (P&U Cisplatin, Pharmacia and Upjohn) stock solution 1 mg/ml, the topoisomerase II inhibitor etoposide (Vepesid, Bristol-Myers Squibb) stock solution 20 mg/ml, the microtubule inhibitor vinblastine (Vinblastine PCH, Pharmachemie) stock solution 1 mg/ml. The methylxanthine derivative, pentoxifylline (Trental, Hoechst, Germany) stock solution 20 mg/ml. Cisplatin and etoposide were stored at room temperature (21 °C), vinblastine and pentoxifylline at 4 °C. Fresh stock solutions were used for each experiment. The criteria for drug selection were clinical potential and suitability for use in cell culture without microsomal activators.

Proliferation arrest of prostatic cells by 5-α DHT

To determine the hormone status of the three prostate cell lines, 4000 cells were seeded in 24-well multiwell plates with medium containing charcoal-stripped (cs) FBS. The medium for the DU145 and BM1604 cell lines was supplemented with 10% csFBS, and 5% csFBS for the LNCaP cell line. 5-α-Dihydrotestosterone (DHT) was added in concentrations ranging from 0.001 nM to 100 nM, for a period of 24 h. The experiment was stopped after 4 days and the cell growth determined by crystal violet assay as described in Baker et al. [1]. Two control flasks were set up for each cell line, 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.

Radiation-induced G_2/M arrest, and abrogation by pentoxifylline

The time of maximum G_2/M phase arrest and G_2 block abrogation was established by flow cytometry, as previously described [2].

Immunochemical detection of $p21^{WAF1}$

The immunochemical detection of $p21^{WAF1}$ was according to a modified protocol of Deptala et al. [7], as previously described [2].

Radiosensitisation effect of pentoxifylline (REF)

Radiosensitisation induced by pentoxifylline (2 mM) added either immediately prior to irradiation, or at the time of maximum G_2 block expression, was assessed by clonogenic survival assay, as previously described [2].

Drug toxicity

The 50% toxicity doses (TD_{50}) for etoposide, cisplatin and vinblastine were determined by crystal violet assay, as previously described [2].

Determination of toxicity enhancement

The influence of G_2 block abrogation on the toxicity of cisplatin, etoposide and vinblastine was assessed by clonogenic survival assays, using the survival of 7 Gy + drug alone (sequential), as controls.

At maximum expression of the G_2 block for DU145, BM1604 and LNCaP (12, 18 and 42 h post-irradiation, respectively), pentoxifylline (at a final concentration of 2 mM) and a cytotoxic drug (at TD_{10}) were added to each flask for a period of 24 h. In a separate set of flasks, the cytotoxic drug was added when the G_2 block was abrogated (8 h after the addition of pentoxifylline), and also exposed for 24 h. The flasks were re-incubated for at least 10 days after a medium change. Colonies were scored and the cytotoxicity enhancement ratio (EF) was calculated thus,

$$EF_1 = \frac{SF\ (7Gy\ +\ drug\ TD_{10})}{SF\ (7Gy + PTX\ +\ drug\ TD_{10}\ at\ G_2\ block)} \quad (1)$$

$$EF_2 = \frac{SF\ (7Gy\ +\ drug\ TD_{10})}{SF\ (7Gy\ +\ PTX + drug\ TD_{10}\ when\ G_2\ block\ is\ abrogated)} \quad (2)$$

Statistics

Data are presented as the mean ± standard deviation of three independent experiments as indicated by error bars (Figs. 1–4) and each experiment was repeated three times. A two-sided *t*-test was used to compare the means between two groups. A *P*-value < 0.05 was considered statistically significant.

Results

Hormone dependence

In the TP53 mutant cell lines, BM1604 and DU145, addition of DHT did not influence cell growth. However, in the TP53 wild-type cell line, LNCaP, DHT concentrations in the range of 0.001–1.0 nM induced a dose-dependent cell proliferation. Increase in the DHT concentration from 10 nM to 100 nM was found to be growth inhibitory (Fig. 1). When LNCaP cells were grown in normal medium it was found that the cells grew significantly better than in charcoal-stripped medium (cs) where the proliferation rate declined to 28%. Supplementing charcoal-stripped medium with 1.0 nM DHT restored cell growth to the level of 55% as compared to normal medium. It can, therefore, be concluded that factors other than steroids were removed by charcoal treatment.

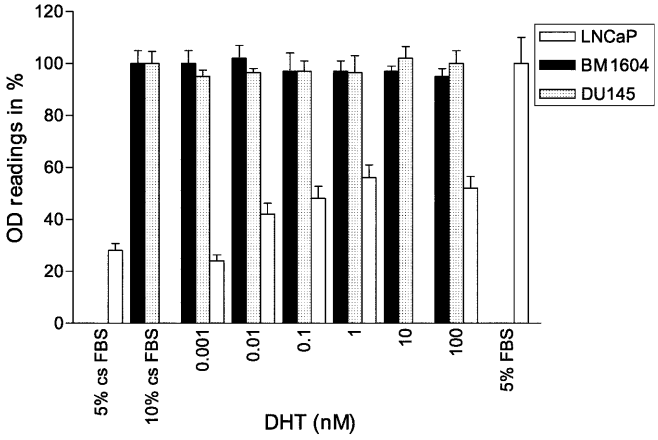

Fig. 1 Proliferation rate of LNCaP, DU145 and BM1604 cells in response to dihydrotestosterone added to charcoal-stripped medium

Induction of p21

In order to demonstrate that the TP53 gene was functional, the TP53 wild-type LNCaP cells were irradiated with 7 Gy and analysed for p21 protein using a p21 antibody and an FITC mouse anti-IgG1 secondary antibody. Figure 2 shows that the p21 level increased from 2 h, reached a peak 4 h post-irradiation and remained high for 24 h. This indicates that the p21 target gene was active and that TP53 was functional. In the DU145 and BM1604 TP53 mutant cell lines no p21 expression could be detected.

Cell doubling times

Doubling times for the three cell lines were: DU145 (40 h), BM1604 (30 h) and LNCaP.FGC (fast growing clone; 42 h).

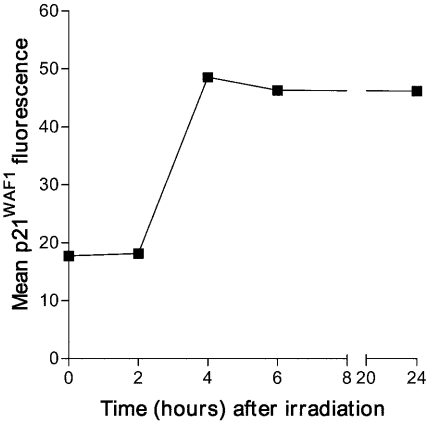

Fig. 2 Induction of $p21^{WAF1}$ protein in TP53 wild-type LNCaP cells in response to 7 Gy ^{60}Co γ-irradiation over a period of 24 h

Influence of irradiation on cell cycle progression

Exposure of prostate cell lines to 7 Gy ^{60}Co γ-irradiation induced cell cycle delays at the G_1 and G_2 cell cycle phases. The TP53 mutant cell lines, BM1604 and DU145, responded to ^{60}Co γ-irradiation damage by arresting in the G_2/M phase. In the TP53 wild-type cell line, LNCaP, a G_1 phase delay, and a smaller G_2/M phase delay was observed (Fig. 3). In the TP53 mutant cell lines, DU145 and BM1604, the G_2/M delay was maximally expressed 12 and 18 h post-irradiation, respectively. The normal recovery time from the cell cycle block was approximately 54 and 66 h post-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 h, with a normal recovery time of approximately 100 h (Fig. 3). Comparison of column 2 to 3 illustrates the progress of normal cell cycle recovery. Comparison of columns 2 and 4 shows the influence of 2 mM pentoxifylline for 8 h. It is apparent that pentoxifylline effectively accelerates cell cycle recovery and, hence, abrogates the G_2/M cell cycle block.

Effect of pentoxifylline alone on radiotoxicity

Radiosensitisation induced by pentoxifylline (2 mM) alone added immediately prior to irradiation was assessed by clonogenic assay in the DU145, BM1604 and LNCaP cell lines, as described elsewhere [2]. The cell line, BM1604, showed a REF of 5.1 for the 7-Gy dose point, compared to a REF of 0.97 when pentoxifylline was added at the G_2 block maximum. When pentoxifylline was present at the time of irradiation in DU145 cells, the REF at 7 Gy was found to be 2.6, 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.

It is apparent that pentoxifylline when added immediately prior to irradiation exerts a radiosensitising effect, particularly in TP53 mutant cells. Radiosensitisation by pentoxifylline was expressed as an enhancement factor (REF), and is given by the ratio of survival fractions (SF), in the presence and in the absence of pentoxifylline.

$$\mathrm{REF} = \frac{\mathrm{SF\ (irradiation)}}{\mathrm{SF\ (irradiation + pentoxifylline)}} \tag{3}$$

Drug toxicity

The 50% toxicity dose (TD_{50}) for pentoxifylline for the three cell lines was found to be 4.2–4.4 mM (not shown). The concentration of 2 mM used for G_2 block abrogation hence is sub-toxic.

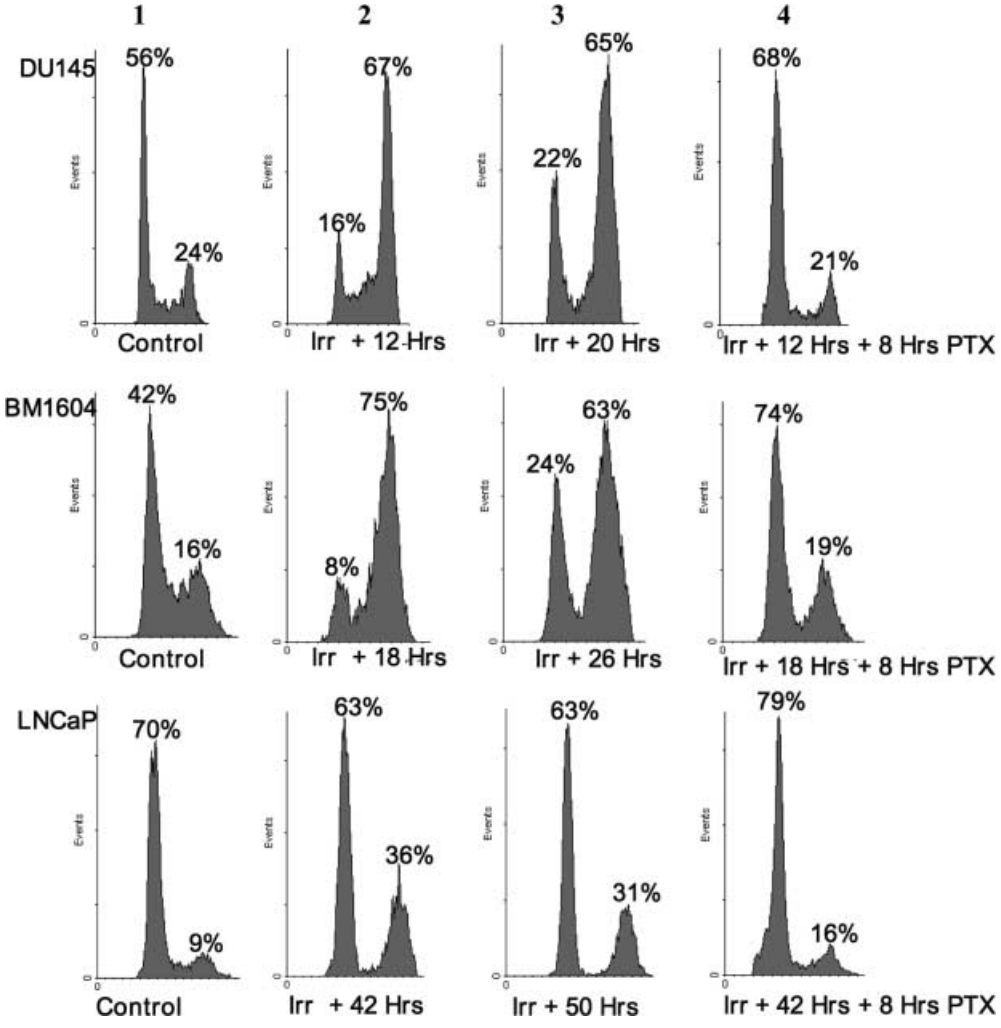

Fig. 3 DNA histograms showing the influence of 7 Gy ^{60}Co γ-irradiation, and 7 Gy ^{60}Co γ-irradiation plus pentoxifylline added at maximum expression of the G_2 block, on the distribution of cells in G_1 and G_2 cell cycle phases at various post-irradiation times in TP53 mutant, DU145 and BM1604, cells, and TP53 wild-type, LNCaP, cells

The 50% toxicity doses (TD_{50}) for vinblastine, cisplatin and etoposide were established by crystal violet assay [2]. Vinblastine emerged as the most toxic drug with TD_{50} values ranging from 2.93 nM to 4.11 nM. Cisplatin took an intermediate position with TD_{50} values ranging from 0.77 μM to 8.20 μM. Etoposide emerged as the least toxic drug with TD_{50} values ranging from 0.80 μM to 9.50 μM (Table 1). It is apparent that the mutant cell lines, DU145 and BM1604, are more drug sensitive than the wild-type cell line, LNCaP. Cell survival curves served to derive the 10% toxic dose (TD_{10}) listed in Table 1 and used to study the influence of G_2 block abrogation on drug toxicity.

Influence of G_2 block abrogation on the toxicity of cisplatin, etoposide and vinblastine

Prostate cells were irradiated with 7 Gy ^{60}Co γ-irradiation, stained with propidium iodide (PI), and the DNA content was determined by flow cytometry. At maximum expression of the G_2 block, 2 mM pentoxifylline and a TD_{10} dose of the anticancer drug were added, and cell survival was determined, using the survival of 7 Gy and drug alone, as controls. It was found that addition of the drug and pentoxifylline at the G_2 maximum, and 8 h later, markedly enhanced the toxicity (Fig. 4). In the TP53 wild-type cell line,

Table 1 TD_{50} and TD_{10} drug concentrations for three human prostate cancer cell lines

Cell lines	Vinblastine		Cisplatin		Etoposide	
	TD_{50}	TD_{10}	TD_{50}	TD_{10}	TD_{50}	TD_{10}
DU145 (TP53 mutant)	3.46 nM	1.26 nM	1.15 μM	0.31 μM	0.80 μM	0.15 μM
BM1604 (TP53 mutant)	2.93 nM	0.77 nM	0.77 μM	0.16 μM	0.82 μM	0.19 μM
LNCaP (TP53 wild-type)	4.11 nM	0.51 nM	8.20 μM	1.20 μM	9.50 μM	1.30 μM

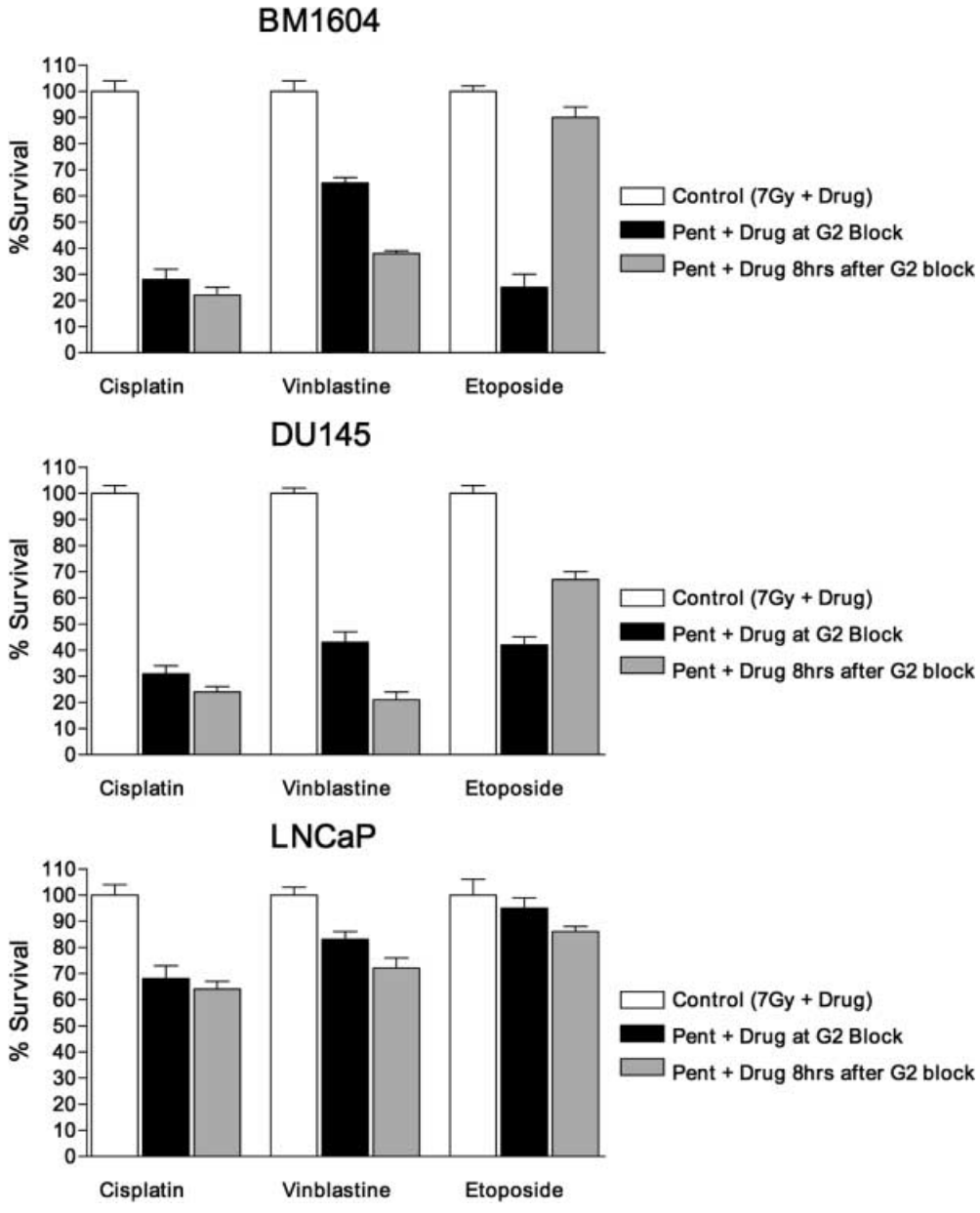

Fig. 4 The influence of cisplatin, vinblastine and etoposide on cell survival in TP53 mutant (DU145 and BM1604) and TP53 wild-type (LNCaP) prostate cancer cell lines under conditions of G_2 block abrogation. Survival at 7 Gy + drug alone served as the control. Drug was added at a toxic dose of 10% (TD_{10}). Pentoxifylline was added at maximum G_2 block expression

LNCaP, increase of drug toxicity was small, showing EFs of 1.10, 1.21 and 1.47, respectively, for etoposide, vinblastine and cisplatin, when the drugs were given at the G_2 block maximum. When the drugs were given 8 h after the G_2 block maximum, EFs were found to be higher, showing values of 1.50, 1.38 and 1.57, respectively, for etoposide, vinblastine and cisplatin (Tables 2, 3). In the mutant cell lines, DU145 and BM1604, the toxicity enhancement factors 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 drug was added at the G_2 maximum (Table 2). When the drug was added 8 h after the G_2 maximum, toxicity enhancement factors for DU145 and BM1604 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 (Table 3).

Table 2 Dose enhancement factors (EFs) for a TD_{10} dose of cisplatin, vinblastine and etoposide, added at maximum expression of the G_2/M block

Treatment	DU145	BM1604	LNCaP
7 Gy Irrad + 2 mM PTX + cisplatin	3.30	3.60	1.47
7 Gy Irrad + 2 mM PTX + vinblastine	2.30	1.53	1.21
7 Gy Irrad + 2 mM PTX + etoposide	2.40	4.00	1.10

Table 3 Dose enhancement factors (EFs) for a TD_{10} dose of cisplatin, vinblastine and etoposide, added 8 h after maximum expression of the G_2/M block

Treatment	DU145	BM1604	LNCaP
7 Gy Irrad + 2 mM PTX + cisplatin	4.11	4.50	1.57
7 Gy Irrad + 2 mM PTX + vinblastine	4.82	2.60	1.38
7 Gy Irrad + 2 mM PTX + etoposide	1.50	1.00	1.50

Discussion

We have demonstrated that irradiation induces a G_2 block in TP53 mutant cells (Fig. 3). This is in agreement with published data [16, 18, 20]. In TP53 wild-type cells irradiation induces a G_1 and a G_2 block, most cells residing in the G_1 phase and a much smaller proportion residing in the G_2 phase [3]. While the onset of the G_2 block is dose independent, the duration (and recovery time) is dose dependent. The addition of pentoxifylline abrogates the G_2 block in TP53 mutant cells as indicated by the very rapid decline of the G_2 population with time and restoration of S-phase (Fig. 3) and published data [3, 16, 20]. The addition of pentoxifylline to G_1-blocked cells also reduces the G_2 population and increases the G_1 population but the DNA synthesis remains suppressed [3]. This has led us to conclude that the addition of pentoxifylline to irradiated TP53 wild-type cells promotes the G_2 to G_1 transition but does not effectively restore the cell cycle, i.e. G_1 to G_2 transition [3].

We have examined the toxicity of etoposide, cisplatin and vinblastine:

1. When cells recover normally from a G_2/M block.
2. When the process is abrogated by pentoxifylline.

The drugs in question were administered after establishment of a G_2 block at essentially non-toxic concentrations (TD_{10}) together with 2 mM pentoxifylline to accomplish G_2 block abrogation. The concentration of pentoxifylline was also kept at a sub-toxic level to avoid any influence on cell survival. Table 2 shows 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 in TP53 mutant cells, is in line with results on TP53 mutant squamous cell carcinoma (scc) and melanoma cells, where the toxicity of cisplatin, melphalan and daunorubicin was found to be up to 80 times more effective [2]. The results on prostate cell lines and the three drugs also show that early entry into mitosis renders these cells particularly drug sensitive, e.g. to cisplatin, vinblastine and etoposide. 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 (approximately 20%) enters the G_1 phase. The lower toxicity enhancements observed in TP53 wild-type cells may be due to the lower population of G_2-blocked cells.

Etoposide has been shown to operate in the G_2 phase of the cell cycle [4]. In our experiments, toxicity enhancements (Table 2) were indeed found to be higher at this point in the cell cycle. The action of cisplatin is independent of cell cycle phases [9] and the toxicity is high in the TP53 mutant cell lines, particularly in the post-G_2 phase (Table 3). Vinblastine prevents microtubule assembly required for cell division and hence arrests cells in the late G_2/M phase [9]. Therefore, a marked toxicity enhancement would be expected under conditions of G_2 block abrogation (Table 3).

It is interesting to note that BM1604 cells show considerable radiosensitivity and an REF of 5.0 at 7 Gy against pentoxifylline alone. This observation highlights the critical importance of irradiation dose and time of drug addition. In a therapeutic application G_2 blocks would arise from sequential 2-Gy dose fractions. At 2 Gy the REFs for pentoxifylline alone were found to be 1.3, 1.4 and 1.0 for BM1604, DU145 and LNCaP, respectively. From the very low REFs shown by pentoxifylline alone at the G_2 maximum and the higher REFs shown when pentoxifylline is added immediately before irradiation, it can be concluded that the mechanism of action is not the same. Early addition of the methylxanthines has generally been linked to repair inhibition, and the reduction of recovery ratios supports this view [21]. Residual unrepaired double-strand breaks remaining after high dose irradiation also strongly suggest that repair inhibition plays a role and must be one mechanism by which pentoxifylline sensitises cells to irradiation [19]. 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 [16]. That this scenario is not toxic to cells could be due to the fact that repair takes place within 2–12 h [8], 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 [10]. It is shown here that prostate cells responded differently to androgen, and show saturation kinetics (Fig. 1). At a concentration of 10^{-12}–10^{-9} M DHT cells undergo active proliferation. At 10^{-7} M the hormone is growth inhibitory and cells undergo growth arrest [11, 13]. The role of androgen in prostatic epithelial cell proliferation is still not fully understood. In cultures of benign prostatic epithelial cells, androgen is not considered a direct mitogen [14]. 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.

Conclusions

The chemosensitivity of prostate tumour cell lines in vitro can be enhanced when the drug is given under conditions of G_2 block abrogation. Irradiated TP53 mutant and androgen independent cells can be sensitised to a subsequent toxic intervention. This process is time dependent and requires that cells have been irradiated to induce a G_2 block. The time dependence of drug toxicity enhancement is also influenced by the pharmacological action of the drugs. The slow development of prostate cancer offers opportunities 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. The fact that toxicity enhancements are observed at low drug doses is particularly encouraging for accomplishing tumour cytostasis without undue systemic toxicity.

Acknowledgements This work forms part of an MSc thesis by AMS. Work was supported by grants from the Cancer Association of SA (CANSA), the National Research Foundation (NRF) of South Africa, and the David and Freda Becker Trust to LB. We thank Professor C.F. Heyns for comments.

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