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

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Abstract Chemotherapeutic drug resistance remains a significant obstacle in the control of prostate cancer. The influence of p53 and androgen status on the drug response of new cell lines from normal, benign and primary tumour epithelium was investigated. The prostate cell lines 1542-NPTX, BPH-1, 1542-CP_3TX, 1532-CP_2TX, 1535-CP_1TX and LNCaP were exposed to TD_{50} doses of etoposide, vinblastine and estramustine for a period of 24 h and re-incubated for a further 4 days before measuring the cell viability by crystal violet vital dye staining assay. The virus-transformed cell lines were found to be approximately ten times more sensitive to etoposide and vinblastine than the non virus-transformed LNCaP cell line. Estramustine proved to be the least toxic drug. The LNCaP cell line emerged as DHT-sensitive against nanomolar concentrations of 5α-dihydrotestosterone in charcoal-stripped growth medium. The virus-transformed cell lines were DHT-insensitive. Induction of p21 by ^{60}Co γ-irradiation was used to assess the functionality of the p53 gene. p21 induction in the LNCaP cell line reached a peak 7.5 h post-irradiation. No significant p21 induction occurred in the virus-transformed cell lines. We show that the androgen-independent tumour cell lines are more sensitive to etoposide and vinblastine than the androgen dependent cell line, LNCaP. Except for LNCaP cells, etoposide and vinblastine were found to be three- to ten-fold more effective than estramustine. In the benign hyperplasia cell line, BPH-1, only etoposide is highly effective. Etoposide and vinblastine were found to effectively inactivate the androgen-independent cell lines, in which p53 is dysfunctional.

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Introduction

The recent success of chemotherapy for the control of prostate tumours [18, 34, 46] has challenged the scepticism concerning chemotherapy for this category of tumours [37, 45]. Retardation of advanced disease by drugs raises the possibility of improved therapy and lower mortality [41]. However, drug resistance remains a major obstacle and is, therefore, a clinically relevant issue.

Historically, the multidrug resistance (MDR) phenotype was believed to be due exclusively to P-glycoprotein overexpression [40]. Use of drug resistant cancer cell lines have identified several alternative mechanisms of drug resistance, namely: (a) altered DNA repair [15, 32], (b) inhibition of apoptosis [13, 24], (c) changes in intracellular drug targets [14, 23] and (d) reduced intracellular concentration of anti-cancer drugs [7, 25]. Whether these mechanisms occur sequentially or concurrently is not known [8].

It is established, however, that the p53 tumour suppressor gene controls a variety of target genes, for example $p21^{WAFI/CIP1}$, MDM 2, bax and Gadd45. These gene products are critical for activation of cell cycle checkpoints, apoptosis and DNA repair. In response to genotoxic stress, wild-type p53 exerts antiproliferative effects such as induction of cell cycle arrest and apoptosis [29]. Inactivation of p53 results in genomic instability [28], and cells either fail to arrest in G_1, exhibit diminished apoptosis [30] or display resistance to chemotherapeutic agents [26]. Considerable effort has been made to examine the correlation between the effects of p53 inactivation and the response of cancer cells to therapeutic drugs [6]. The results have been conflicting, with some studies indicating enhanced sensitivity and others indicating increased resistance to the same drugs (reviewed in [4, 21]).

As far as control of prostate cancer is concerned it is not clear at present why established drugs such as

vinblastine, estramustine and etoposide work better in some patients than in others, and more specifically how tumour response is influenced by p53 status and androgen dependence. The cell biological approach to this problem has been complicated by the fact that existing prostate tumour cell lines such as DU145, LNCaP and PC3 are derived from metastatic tissue and hence may not be clinically representative. The availability of a panel of cell lines from benign and primary prostate tumour epithelium and from normal prostate tissue prompted us to assess the influence of p53 status, p53 functionality and androgen dependence on drug sensitivity. We show that loss of p53 function and androgen independence is not correlated with tumour resistance to vinblastine, estramustine and etoposide.

Material and methods

Cell culture

The epithelial cell line, benign prostatic hyperplasia-1 (BPH-1), was established from human prostate tissue obtained by transurethral resection, and immortalised with the large T gene of the simian virus 40 (SV40) [16]. BPH-1 grows in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), penicillin (100U/ml) and streptomycin (100 μg/ml) (Highveld Biological, South Africa). The culture was obtained from Prof. S.W. Hayward (Department of Urology, University of California, USA).

The malignant 1535-CP_1TX, 1532-CP_2TX and 1542-CP_3TX prostate epithelial cell cultures (1532T, 1535T and 1542T), and the normal 1542-NPTX prostate epithelial cell culture (1542N), were derived from primary adenocarcinomas resected from six patients, and immortalised with the E6 and E7 genes of the human papilloma virus 16 (HPV-16) [5]. They were grown in WAJC 404 medium (Gibco BRL, Scotland, UK) containing hydroxyethylpiperazine ethanesulfonic acid, sodium hydrogen carbonate, zinc stabilised insulin, cholera toxin, dexamethazone, epidermal growth factor (Sigma-Aldrich, Germany), penicillin/streptomycin and 0.5% heat-inactivated FBS (Highveld Biological, SA), as described elsewhere [2]. The cultures were provided by Prof. J.R.W. Masters (Prostate Research Centre, University College London, UK) and Prof. J.A. Macoska (Department of Urology, University of Michigan Comprehensive Cancer Center, USA).

The LNCaP cell line was established from a supraclavicular lymph node metastasis of human prostatic adenocarcinoma [17]. The cells were obtained from Dr. J.H. Visser (Department of Urology, University of Stellenbosch Medical Faculty, South Africa) and were grown in RPMI 1640 medium supplemented with 5% heat-inactivated FBS, penicillin (100U/ml) and streptomycin (100 μg/ml; Highveld Biological).

All cell lines were grown at 37°C in a humidified incubator with 5% CO_2.

Drug and chemical preparation

The microtubule destabilising agent, estramustine (Estracyt 3, Pharmacia and Upjohn), was prepared as a stock solution of 10 mg/ml. The topoisomerase II inhibitor, etoposide (Vepesid, Bristol-Myers Squibb), was prepared as a stock solution of 20 mg/ml. The microtubule inhibitor, vinblastine (Vinblastine PCH, Pharmachemie), was prepared as a stock solution of 1 mg/ml. Estramustine and etoposide were stored at room temperature (21°C), and vinblastine at 4°C. Stock solutions were made fresh for each experiment. The criteria for drug selection are based on clinical potential, and suitability for use in cell culture without microsomal activators.

Drug toxicity

Drug concentrations were determined as follows. Exponentially growing cells were trypsinised and counted, and 10,000 cells/ml seeded into 24-well multiwell plates. The cells were allowed to settle for 5–6 h before the addition of varying concentrations of drug. Working solutions were freshly prepared for each experiment. After 24 h of drug exposure the medium was changed and the multiwell plates re-incubated for a further 4 days. The cells were then fixed with a solution of buffered formalin (pH 7.2) and stained with 0.01% crystal violet. The crystal violet stain was dissolved in 1 ml of a 10% sodium dodecyl sulphate solution overnight and the optical density of the extracted stain read on a Perkin-Elmer Lambda 5 spectrophotometer at 590 nm. Cell survival at each drug concentration was expressed as a percentage of the control survival rate, i.e. where no drug was present in the growth medium, corrected for cell plating efficiency.

Immunochemical detection of p21WAF1

$p21^{WAF1}$ detection was performed according to a protocol modified from Deptala et al. [9]. Cells were fixed in 1% methanol-free formaldehyde in PBS at 4°C for 15 min, washed with PBS and permeabilised with 80% ethanol at –20°C. After fixation and reswelling in PBS at room temperature (22°C) cells were lysed in 0.25% Triton X100 on ice for 5 min. Cells were then washed with PBS containing 1% bovine serum albumin (BSA) and incubated overnight at 4°C with 100 μl 1% BSA/PBS containing 1 μg anti-$p21^{WAF1}$ antibody (PharMingen, clone 2G12, cat. no. 15441A) or 3 μl mouse anti-IgG1 (Dako, X0931) in a 1:30 titre as a negative control. Thereafter, the cells were washed in 1% BSA/PBS and incubated in 100 μl 1% BSA/PBS containing 3 μg mouse anti-IgG1 secondary antibody (Sigma, F-2012) for 1 h in the dark at room temperature. $p21^{WAF1}$ induction was assessed by flow cytometry measuring green fluorescein isothiocyanate (FL1-H) versus red PI (FL3-H) on a fluorescence-activated cell sorter.

Proliferation arrest of prostatic cells by 5-α DHT

A quantity of 10,000 cells were seeded in 24-well multiwell plates with medium containing charcoal-stripped (cs) FBS. The medium for the 1532T, 1535T, 1542T and 1542N cell lines was supplemented with 0.5% csFBS, the BPH-1 cell line with 10% csFBS, and 5% csFBS for the LNCaP cell line. 5α-Dihydrotestosterone (DHT) was added 1 day later (2 days later in the case of LNCaP) in concentrations ranging from 0.001–10 nM, for a period of 4 days. The experiment was stopped after 4 days and the cell growth determined by crystal violet assay as described in Baker et al. 1986 [1]. 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.

Statistics

Data are presented as the mean ± standard deviation of three independent experiments as indicated by error bars (Figs. 1, 2, 3), and each experiment was repeated three times. A two-sided t test was used to compare the means between groups. A P value less than 0.05 was considered statistically significant.

Results

Androgen sensitivity

The crystal violet vital dye staining assay shows that addition of DHT has no significant effect on cell

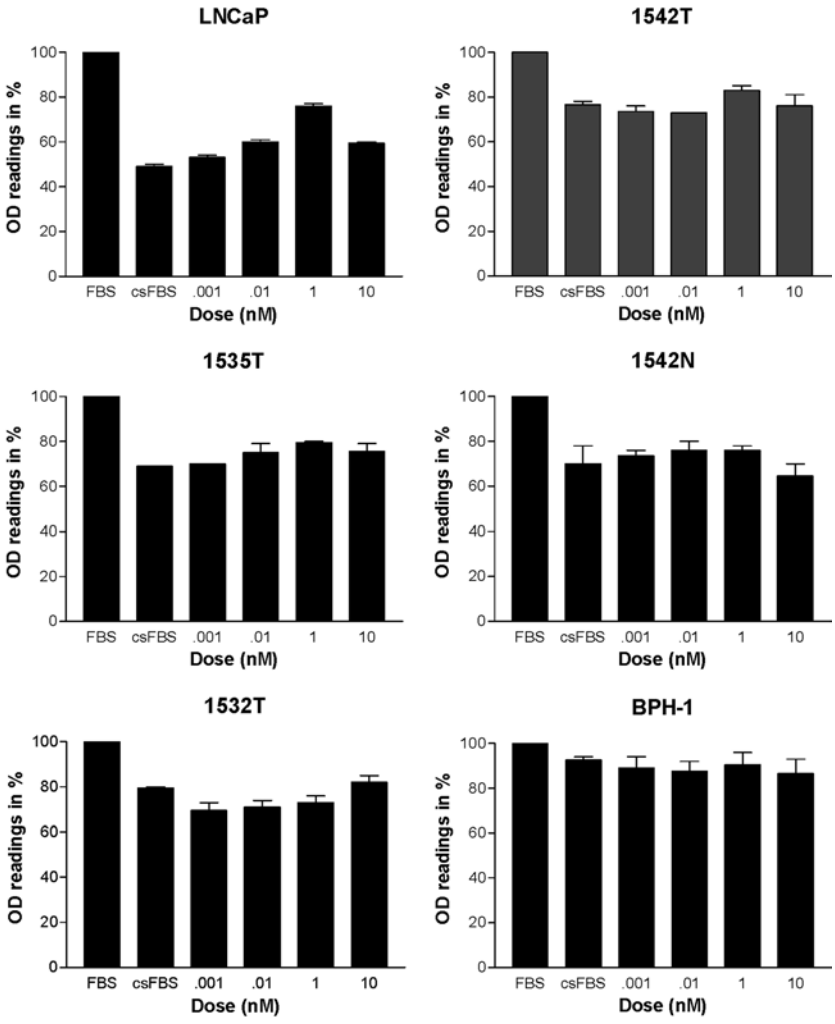

Fig. 1. The effect of DHT addition (nM) to charcoal-stripped medium on the proliferation of six prostate cancer cell lines measured by crystal violet dye staining assay

growth in the 1532T, 1535T, 1542T, 1542N and BPH-1 cell lines (Fig. 1). In these cell lines growth in csFBS equalled or exceeded growth in the presence of 0.001–10 nM DHT, indicating that they are androgen independent. However, when the LNCaP cells were subjected to cs medium, and DHT was then added at concentrations of 0.001–1.0 nM, growth was restored from 50% in csFBS to a level of 80% of control (FBS) (Fig. 1). This confirms that LNCaP cells are androgen dependent. The fact that DHT did not restore growth to 100% indicates that factors, other than steroids, removed by charcoal treatment, were essential for optimal growth.

Growth characteristics

The doubling times for the malignant cell lines 1532T, 1535T and 1542T were found to be in the range of 28–29 h. As expected, the normal cell line 1542N has a longer doubling time (36 h) whereas the benign BPH-1 cell line has a very short doubling time (16 h) (not shown).

Induction of p21

Induction of $p21^{WAF1}$ gene by ^{60}Co γ-irradiation was used to assess p53 function. The p53 wild-type cell line, LNCaP, expresses functional p53 as indicated by the induction of the p21 target gene (Fig. 2). No appreciable

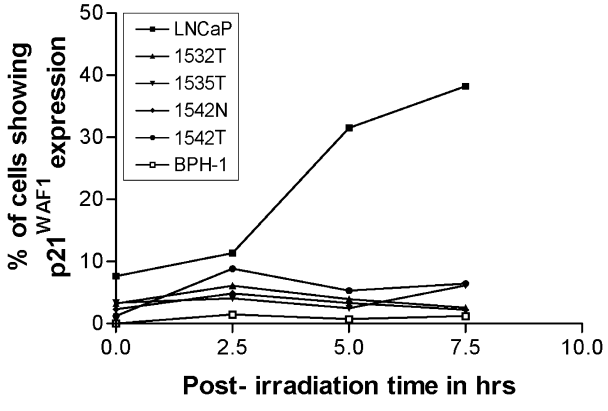

Fig. 2. The response of prostate cell lines to 7 Gy ^{60}Co γ-irradiation in terms of nuclear accumulation of $p21^{WAF1}$

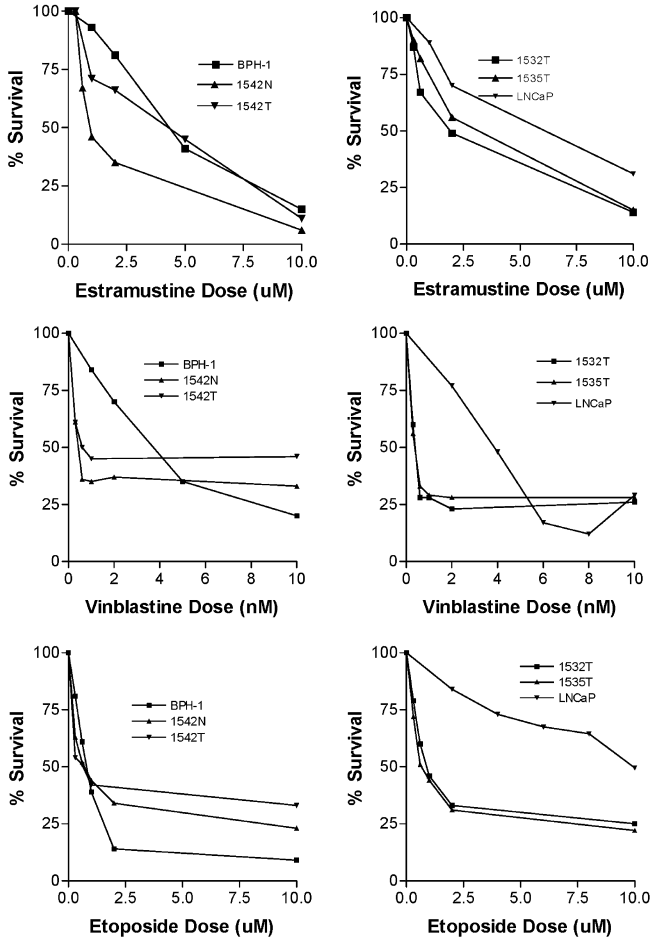

Fig. 3. Dose response curves of estramustine, vinblastine and etoposide for six human prostate cell lines

p21 induction was apparent during the initial 2.5 h post-irradiation but p21 induction reached a peak 7.5 h post-irradiation (7 Gy ^{60}Co γ-irradiation). No significant p21 induction was evident in the 1532T, 1535T, 1542T and 1542N cell lines immortalised with the E6 and E7 transforming genes of HPV-16, or the BPH-1 cells which have been immortalised with the large T antigen gene of SV40.

Drug toxicity

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

- Etoposide:
  LNCaP > BPH > 1532T > 1535T > 1542N > 1542T
- Vinblastine:
  LNCaP > BPH > 1532T > 1542N > 1535T > 1542T
- Estramustine:
  BPH > LNCaP > 1535T > 1542N > 1532T > 1542T

It is apparent that LNCaP is the most resistant cell line against vinblastine and etoposide, and the second most resistant against estramustine. The 1542T cell line is by far the most sensitive cell line against all 3 drugs (Fig. 3, Table 1). Vinblastine is the most toxic drug with TD_{50} values ranging from 0.15 to 3.70 nM. Etoposide takes an intermediate position with TD_{50} values ranging from 0.15 to 4.70 μM. Estramustine is the least toxic drug shown by TD_{50} values ranging from 1.45 to 4.30 μM.

Discussion

The human prostate cell lines DU145, PC3 and LNCaP originate from metastatic lesions [17, 19, 44]. These cell lines are in common use and represent the most widely used cell biological model. A model which is closer to clinical reality would be epithelial prostate cultures which reflect the in situ characteristics of normal epithelium, the epithelium of benign hyperplasia, and the malignant epithelium [5, 16]. Such cell lines were used in this study.

The cell lines grow in the absence of FBS, with or without DHT supplementation, at rates comparable to that in the presence of serum. It is apparent in Fig. 1 that LNCaP cells respond to DHT stimulation and hence are androgen dependent. This is consistent with published data [17]. It is also evident (Fig. 1) that all the transformed cell lines are androgen independent. The differences in growth between the FBS cultures and the csFBS cultures (Fig. 1) may be attributed to the removal of substances which are essential for growth. This is in agreement with the observation that the addition of the synthetic androgen, methyltrienolone [5] can restore cell growth after stripping of the growth medium on charcoal.

As expected, cell inactivation was drug and concentration dependent (Fig. 3, Table 1). The differential response of cell lines to the same drug could be due to multidrug resistance (MDR). The following patterns can be distinguished: the 1542T cell line is the most chemosensitive, and the LNCaP cell line is the most chemoresistant. We speculate that the low chemoresistance of LNCaP cells against estramustine, as compared to BPH-1 cells, may be due to an androgen-suppressing effect of this drug. Interestingly, the 1542N cell line appears more resistant to vinblastine, etoposide and estramustine than its tumour counterpart 1542T (Fig. 1). Unlike Berchem et al. [3] and Raffo et al. [35] we do not find that the transition of prostate cancer cells to a

Table 1. TD_{50} drug concentrations for 6 human prostate cell lines

Cell lines	Etoposide (μM)	Vinblastine (nM)	Estramustine (μM)
LNCaP	4.70	3.70	3.90
BPH-1	0.70	3.34	4.30
1532T	0.57	0.29	1.76
1535T	0.45	0.26	2.51
1542T	0.15	0.15	1.45
1542N	0.39	0.28	1.85

hormone-independent state leads to chemoresistance. Our results clearly demonstrate that the hormone-independent cell lines are the most chemosensitive (Fig. 3, Table 1). Estramustine proved to be the least toxic agent. This is in agreement with other studies showing that this drug has no significant single-agent activity in hormone-refractory prostate cancer [34, 43]. However, there is in vitro evidence that a combination of estramustine and taxol, or estramustine and etoposide has a supra-additive cytotoxic effect on prostate cancer cells [33, 43].

Androgen treatment of the hormone-dependent human LNCaP cells has been shown to induce chemoresistance [3]. In a previous study on a panel of prostate cell lines we have shown that chemoresistance is linked to androgen dependence [39].

Nearly all drug-induced cell inactivation invokes apoptosis [36]. Model systems have demonstrated that factors which influence the apoptotic pathway can confer relative resistance to cytotoxic agents [20]. In this scenario the apoptosis inhibiting bcl-2 gene product [12, 27, 36] would be of interest, but such an analysis was beyond the scope of this investigation. A major factor in the cellular damage response is the p53 tumour suppressor gene. Evidence for a link between the growth suppressing activity of p53 and the inactivation of cyclin-dependent kinases has been provided by the cloning of the WAF1/CIP1 gene (p21), the transcription of which is directly activated by p53. Expression of WAF1/CIP1 in mammalian cells inhibit cell growth, suggesting that p21 is a downstream mediator of p53 function [10]. It has also been shown that WAF1/CIP1 is induced by DNA damaging agents that trigger G_1 arrest or apoptosis in cells with wild-type p53 but not in tumour cells harbouring deletions or mutations in the p53 [11]. Michieli et al. [31] have suggested the existence of two separate pathways for the induction of WAF1/CIP1, a p53-dependent one elicited by DNA damage which leads to growth arrest, and a p53-independent one triggered by growth factors associated with cell growth. It is clear therefore that p53 status is important and that factors which influence the functionality of this gene need to be considered.

The 1542T, 1542N, 1532T and 1535T cell lines were obtained by HPV-16 transduction and introduction of the E6 and E7 transforming genes which results in p53 degradation and suppression of negative growth signals emanating from p53 [42]. The BPH-1 cell line has been transformed with the SV40 large T antigen, which renders the p53 protein dysfunctional as the result of sequestration to oncogene products [38]. It is therefore not surprising that only the non virus-transformed cell line LNCaP expressed functional p53 as indicated by the induction of p21 (Fig. 2).

Recent data on Wi38VA13 cells have shown that p53 can be functional in certain types of SV40 transformed cells [47]. An important conclusion of the p21 data is that all virus-transformed cell lines (BPH-1, 1532T, 1535T, 1542T and 1542N) show complete abolition of p53 function and not partial p53 inactivation by incomplete complexation to the virus product.

The conclusion of the p21 controls and p53 functionality tests are that the p53-defective cell lines emerge with superior sensitivity to vinblastine and etoposide. This is evident from the low TD_{50} data for etoposide and vinblastine (Table 1) which demonstrate that the p53-defective cell lines are approximately ten times more drug sensitive than the LNCaP line in which p53 is intact.

The finding of superior drug sensitivity in p53-defective prostate cell lines is in contrast to results on human colon cancer cell lines which have shown that p53 disruption rendered cells resistant to the antimetabolite, 5-fluorouracil [6]. However, a definitive conclusion of the influence of the p53 degrading viral proteins is not possible because it appears that the E6 protein has effects other than those mediated by p53 inhibition [22].

The androgen-dependent tumour line LNCaP emerges as ten times less sensitive to vinblastine and etoposide. It is furthermore indicated that estramustine does not distinguish benign hyperplasia cells from prostate tumour cell lines. Regrettably, most of the cell lines investigated here were androgen independent. Availability of a p53-defective androgen dependent tumour cell line could have expanded the drug sensitivity assessment even further.

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