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# Microarray analysis of bicalutamide action on telomerase activity, p53 pathway and viability of prostate carcinoma cell lines

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## Abstract

Bicalutamide is a non-steroidal anti-androgen commonly used in the treatment of prostate carcinoma. We analysed the transcriptional response to bicalutamide treatment with the aim of explaining the inhibition of telomerase in the androgen-sensitive cell line LNCaP and the effects of bicalutamide on the androgen-insensitive cell line DU145. Cells treated with 80 $\mu$M bicalutamide in steroid-depleted medium for 1 day were analysed in duplicate by Affymetrix Human Genome Focus Arrays. Response to bicalutamide in LNCaP cells was represented by downregulation of androgen-regulated genes, activation of the p53 pathway and inhibition of telomerase, which was associated with downregulation of v-myc avian myelocytomatosis viral oncogene homologue (MYC) and telomerase reverse transcriptase subunit. In DU145 cells we observed the influence of cell density on bicalutamide effectivity such that highly confluent cells showed lesser sensitivity than low confluent ones. In conclusion, we provide an explanation for telomerase inhibition after androgen receptor blockade in LNCaP cells and we also report activation of the p53 pathway in LNCaP cells and in-vitro sensitivity to bicalutamide of low confluent androgen-insensitive DU145 cells. These findings might have implications for both experimental and clinical research into prostate cancer. In particular, activation of the p53 pathway after treatment with 80 $\mu$M bicalutamide could justify usage of bicalutamide dosages higher than 150 mg daily in androgen-sensitive carcinoma therapy.

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## Introduction

Prostate cancer is one of the most common cancers of European and North American men. At present, androgen deprivation is the only effective systemic therapy available for advanced prostate cancer. The inability of androgen deprivation to completely and permanently eliminate all prostate cancer cell populations is manifested by relapse, with the ultimate progression to androgen independence (Denis & Murphy 1993).

Bicalutamide (Casodex, AstraZeneca) is a non-steroidal anti-androgen with a favourable tolerability profile that represents an alternative therapeutic strategy to castration (Furr et al 1987; Schellhammer 2002). Currently, 50 mg bicalutamide daily is an appropriate dose to use in combined androgen blockade while 150 mg is being evaluated as a suitable dose for monotherapy (Kolvenbag & Nash 1999). Long-term administration of 50 mg per day was reported to result in a mean plasma concentration of 21.7 $\mu$M of bicalutamide (Cockshott et al 1990). Monotherapy of 150 mg daily resulted in a mean plasma concentration of 50.2 $\mu$M (range 30.9–104.8 $\mu$M; Tyrrell et al 1998). Kolvenbag et al (1998) did not observe increases in pharmacological side-effects or other adverse events in doses up to 600 mg daily. The decreases in prostate-specific antigen (PSA) with these doses were comparable to that observed with 150 mg or with castration.

Telomerase is a specialized ribonucleoprotein polymerase that adds hexameric repetitive sequences (TTAGGG) onto human chromosomal ends using a segment of its integral RNA component as a template (Blackburn 1992). Normal human somatic cells express low or undetectable telomerase activity and progressively lose their telomeric sequences, which results in replicative senescence and ageing. In contrast, telomerase activity is

present in germ-line cells and in the majority of stem and cancer cells (Harley 1991). Knowledge of the regulation of telomerase activity has relevance not just for an understanding of tumour biology in general but might also provide implications for specific anti-telomerase therapy.

In the present study we analysed telomerase activity and global gene response after bicalutamide treatment in both androgen-sensitive (LNCaP) and -insensitive cell lines (DU145). We used Affymetrix Human Genome Focus Arrays comprising over 8500 probe sets of human genes with the aim of further explaining our preliminary results describing the telomerase and growth inhibition mediated by bicalutamide (Bouchal et al 2002). In contrast to the previous work we shortened the treatment interval from 3 days to 1 day and raised the concentration of bicalutamide from 50 to 80 $\mu$M in order to obtain early and more pronounced gene responses.

## Materials and Methods

### Cell culture and cell viability assay

The LNCaP and DU145 cells lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD). The cell lines were maintained as recommended by ATCC and experiments were performed in RPMI 1640 supplemented with 2.5% (v/v) charcoal-stripped FBS. Cells were cultivated in this steroid-free medium for 1 day before any treatment. The MTT cell viability assay (Romijn et al 1988) was used to determine the concentration of bicalutamide inhibiting cell viability to 50% ($IC_{50}$). In brief, cells in quadruplicate were exposed to bicalutamide for 3 days in 96-well plates, then incubated with MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), which was converted by viable cells to blue formazan. The absorbance was measured at 540 nm after lysis in 10% (w/v) sodium dodecyl sulfate (SDS).

### Microarray analysis

Total cellular RNA was isolated from approximately $5\times10^6$ cells using Trizol (Invitrogen, CarlsBad, CA) according to the manufacturer's recommendations and subsequently cleaned by Rneasy Mini Kit (Qiagen, Hilden, Germany). For biotin-labelled target synthesis, reactions were performed using standard Affymetrix protocols. Total RNA (10 $\mu$g) was converted into double-stranded cDNA by reverse transcription using T7-(dT)24 primer (Helena Biosciences, Sunderland, UK) and cleaned by a GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA). After second-strand synthesis, biotin-labelled cRNA was generated from the cDNA sample by an in-vitro transcription reaction using an Enzo BioArray HighYield RNA Transcript Labelling Kit (Affymetrix). After purification by a GeneChip Sample Cleanup Module (Affymetrix), the concentration of labelled cRNA was determined by UV absorbance measurement. In all cases, 25 $\mu$g of each biotinylated cRNA preparation was fragmented, assessed by gel electrophoresis, and placed in a hybridization cocktail containing biotinylated hybridization controls (GeneChip Eukaryotic Hybridization Control Kit, Affymetrix). Samples were first hybridized to Test3 Arrays for 16 h, then washed, stained using antibody-mediated signal amplification and scanned. After this quality control, the samples were hybridized onto the larger Human Genome Focus Arrays. The images from scanned chips were processed using Affymetrix Microarray Analysis Suite 5.0 (see the Statistical analysis section). The generated absolute and comparison data were then uploaded onto an Affymetrix Micro DB database and processed with Affymetrix Data Mining Tool 3.0 software. The gene lists were then transferred to Microsoft Excel and analysed by NETAFFX Analysis Center (Affymetrix), which provides links to internet genome databases (Gene Ontology database, GeneCards, Swiss prot, etc.).

### Telomerase activity

Telomerase activity was analysed by a TRAPeze kit (Chemicon, Temecula, CA) based on the TRAP (Telomeric Repeat Amplification Protocol) method (Kim et al 1994). In brief, the concentration of proteins in cell lysates was measured by the Bradford method and aliquots of the lysate containing 1 $\mu$g of protein were loaded for the primer elongation and PCR. After separation on 12% polyacrylamide gel, the telomerase ladder was visualised by Sybr Gold and charge coupled device (CCD) camera (Raytest, Straubenhardt, Germany). The telomerase activity was measured in triplicate for both cell lines at several time intervals after bicalutamide treatment.

### Real-time quantitative RT-PCR

Quantification of telomerase reverse transcriptase (TERT) transcript was performed by Light Cycler Telo-TAGGGhTERT Quantification kit (Roche Diagnostics, Basel, Switzerland; information on primers and probes was not provided). Analysis was carried out according to the manufacturer's protocol with 150 ng of RNA, from the same isolates used in the microarray analysis. For each sample, the amount of TERT transcripts was divided by the amount of house-keeping porphobilinogen deaminase (PGBD) transcripts. The resulting ratio therefore showed the TERT value normalized to the expression of the house-keeping gene. Quantification of TERT mRNA was performed only for those samples analysed by microarrays.

### Immunoblotting

Cultured cells were detached by trypsin, immediately re-suspended in SDS loading buffer and boiled for 5 min. After separation by electrophoresis, proteins were transferred onto nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK). The membranes were stained with Ponseau Red (Sigma, St Louis, MO) for control of sample loading. After blocking in buffer containing 5% (w/v) milk, the membranes were treated overnight at 4°C with appropriate antibody (see below). Following a second incubation with peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies (Santa Cruz Biotechnology, Santa

Cruz, CA), the proteins were visualized with Supersignal West Pico/Dura Chemiluminescent Substrate (Pierce, Rockford, IL). The antibodies used were rabbit polyclonal anti-caspase-3 (H-277, Santa Cruz) and mouse monoclonal anti-c-Myc (clone Ab-2, Calbiochem, Darmstadt, Germany), anti-androgen receptor (clone AR441, Santa Cruz), anti-$p21^{waf1/cip1}$ (clone 118, kindly provided by Dr Vojtesek), anti-p53 (DO-1, Dr Vojtesek) and anti-poly(ADP-ribosyl) polymerase (PARP, C2–10, Dr Vojtesek). Immunoblotting of selected proteins was performed at least three times in samples submitted to microarray analysis and in verification experiments.

### Statistical analysis

MTT cell viability tests were performed in three independent experiments with quadruplicate estimation in each of them. The effects of bicalutamide concentration (25, 50, 60, 70, 80, 100, 120 and 150 $\mu$M) on percentage control for both cell lines (LNCaP, DU145) and confluence (low, high) were statistically analysed by the Kruskal–Wallis test (Statistica 6.0, StatSoft, Prague, Czech Republic). Individual differences between cell lines in both confluences for each concentration were then examined by the Mann–Whitney U-test. Significance levels of $P < 0.05$ and $P < 0.001$ are denoted in Figure 4.

The microarray analysis of 1-day treatment with 80 $\mu$M bicalutamide was performed in duplicate in both cell lines. LNCaP cells were treated in two independent experiments while DU145 cells were treated in duplicate in one experiment with one control. Single analysis of 3-day treatment with the same drug concentration was performed in the LNCaP cell line only as well as 1-day treatment with 1 nM dihydrotestosterone and 10 nM 17$\beta$-estradiol. With respect to the previous results (Bouchal et al 2002), showing that hormone co-treatment does not change the $IC_{50}$ of bicalutamide, we treated cells with bicalutamide and hormones separately. A higher number of replicates would strengthen our conclusions. However, the use of single control and treatment pairs has been discussed previously in the literature (Toda et al 2003). Our conclusions are based on analyses of replicate treatments, further supported by data obtained from single control and treatment pairs. By considering only genes with high fold change we reduce the possibility of incorrect conclusions with respect to high variability caused by small numbers of replicates (Sasik et al 2002).

The statistical analysis of microarray data is described in detail at the Affymetrix website (www.affymetrix.com/support/downloads/manuals/data_analysis_fundamentals_manual.pdf). In brief, the expression levels and detection calls of mRNAs were determined on each array using the absolute analysis algorithm (Microarray Analysis Suite 5.0, Affymetrix). The expression levels were calculated by implemented Tukey's one-step biweight estimate as the weighted mean fluorescence intensity among intensities obtained by 11 to 20 paired (perfectly matched and single nucleotide-mismatched) probes consisting of 25-base oligonucleotides. The absolute analysis algorithm with implemented Wilcoxon's one-sided signed rank test also used probe pair intensities to generate a detection $P$ value and assigned a present, marginal or absent call. The comparison analysis algorithm was used in order to compare gene expression levels between two samples. The analysis employed normalization and scaling techniques to minimize differences in overall signal intensities between the arrays. The data were then analysed using comparison algorithms to derive a different call, which indicates whether a transcript is increased or decreased or exhibits no change in expression level. The Wilcoxon signed rank test was used in comparison analysis to derive biologically meaningful results from the raw probe cell intensities on expression arrays. During a comparison analysis, each probe set on the experiment array was compared to its counterpart on the baseline (control) array, and a change in $P$ value was calculated. The final $P$ values were categorized by cut-off values called gamma 1 ($\gamma_1 = 0.0045$) and gamma 2 ($\gamma_2 = 0.006$), which provide boundaries for change calls: increase ($0 \le P$ value $\le \gamma_1$), marginal increase ($\gamma_1 \le P$ value $\le \gamma_2$), no change ($\gamma_2 \le P$ value $\le 1 - \gamma_2$), marginal decrease ($1 - \gamma_2 \le P$ value $\le 1 - \gamma_1$) and decrease ($1 - \gamma_1 \le P$ value $\le 1$) in gene expression. The magnitude and direction of change of each transcript was estimated by the signal log ratio, which was computed by an implemented Tukey's one-step biweight method taking a mean of the log ratios of probe pair intensities across the two arrays. The log scale used is base 2 and the fold change was calculated for the tables in this article. Full microarray data are available online at www.lmp.upol.cz/affymetrix.

## Results

### Activation of p53 pathway in LNCaP cells

As can be seen from the total numbers of genes responding by increase/decrease to 1-day treatment with 80 $\mu$M bicalutamide, the reaction was stronger in LNCaP cells (619/427) than in DU145 cells (153/161). Genes changed at least five times in LNCaP cells are listed in Table 1. Among these, p53-dependent genes, such as $p21^{waf1/cip1}$, GADD45$\alpha$ and PIG3, were upregulated. Other p53-regulated genes, such as MDM2, BAX and TRAILR2, were increased less than five times (data not shown). The level of p53 mRNA was not changed, but increase of p53 protein as well as $p21^{waf1/cip1}$ protein was confirmed by immunoblotting (Figure 1). Among highly decreased genes were androgen receptor regulated prostate specific antigen, kallikrein 2 and prostatic acid phosphatase. Cell cycle and survival regulating factors, such as survivin, CDC2, CDC20, CDC28 protein kinase, MCM2 and MAD2-like1, were also decreased. The changes in the mRNA level were associated with cleavage of procaspase-3 and PARP protein (Figure 1) as well as with membrane blebbing and cellular fragmentation (Figure 2). Treatment with 50 $\mu$M bicalutamide resulted in similar changes to those seen with the 80 $\mu$M dose but with lower amplitude (Figure 1).

### Telomerase inhibition by bicalutamide in LNCaP cells

Maximal decrease of telomerase activity was observed after 3 days of treatment by bicalutamide in LNCaP

**Table 1** Genes changed at least five-fold after 1 day of treatment with 80 $\mu$M bicalutamide in LNCaP cells

| Affymetrix ID | GenBank ID | Description | Fold ± Int[a] | | Ctrl[b] |
|---|---|---|---|---|---|
| 207154_at | NM_001362.1 | Deiodinase, iodothyronine, type III (DIO3) | 31.3 | 25 | 11[x] |
| 202672_s_at | NM_001674.1 | Activating transcription factor 3 (ATF3) | 13.2 | 3.8 | 137 |
| 217967_s_at | AF288391.1 | Niban protein (C1orf24)†† | 11.7 | 1.6 | 30 |
| 203234_at | NM_003364.1 | Uridine phosphorylase (UP)†† | 11.0 | 0.2 | 6[x] |
| 213112_s_at | N30649 | Sequestosome 1 (SQSTM1) | 10.8 | 2.2 | 28[x] |
| 204351_at | NM_005980.1 | S100 calcium-binding protein P (S100P) | 9.6 | 4.6 | 33[x] |
| 210609_s_at | BC000474.1 | Tumour protein p53 inducible protein 3 (TP53I3) | 7.7 | 0.1 | 44[x] |
| 203725_at | NM_001924.2 | Growth arrest and DNA-damage-inducible, alpha (GADD45A) | 7.6 | 0.3 | 62 |
| 203961_at | AL157398 | Nebulette protein (NEBL) | 7.4 | 0.3 | 54 |
| 202284_s_at | NM_000389.1 | Cyclin-dependent kinase inhibitor 1A ($p21^{waf1/cip1}$) (CDKN1A) | 7.3 | 1 | 213 |
| 221760_at | BG287153 | Mannosidase, alpha, class 1A, member 1 (MAN1A1) | 7.3 | 0.2 | 11[x] |
| 202054_s_at | NM_000382.1 | Aldehyde dehydrogenase 3 family, member A2 (ALDH3A2) | 6.5 | 1.4 | 98 |
| 221050_s_at | NM_019096.1 | GTP binding protein 2 (GTPBP2)† | 6.5 | 0.8 | 56[x] |
| 219863_at | NM_016323.1 | Cyclin-E binding protein 1 (CEB1) | 6.3 | 2.9 | 19[x] |
| 202842_s_at | AL080081.1 | DnaJ (Hsp40) homologue, subfamily B, member 9 (DNAJB9)†† | 6.0 | 0.1 | 118 |
| 221142_s_at | NM_018441.1 | Peroxisomal *trans*-2-enoyl CoA reductase (PECR) | 5.9 | 3.4 | 37[x] |
| 219628_at | NM_022470.1 | Hypothetical protein similar to wild-type p53-induced gene 1 (FLJ12296) | 5.8 | 0.3 | 59 |
| 209921_at | AB040875.1 | Solute carrier family 7, member 11 (SLC7A11) | 5.7 | 0.9 | 120 |
| 205110_s_at | NM_004114.1 | Fibroblast growth factor 13 (FGF13) | 5.5 | 0.8 | 54 |
| 209173_at | AF088867.1 | Anterior gradient 2 (Xenopus laevis) homologue (AGR2) | 5.3 | 0.9 | 63[x] |
| 221156_x_at | NM_004748.1 | Cell cycle progression 8 protein (CPR8)† | 5.3 | 0.1 | 28 |
| 201141_at | NM_002510.1 | Glycoprotein (transmembrane) nmb (GPNMB) | 5.3 | 0.8 | 51 |
| 200924_s_at | NM_002394.1 | Solute carrier family 3, member 2 (SLC3A2)†† | 5.2 | 1.5 | 130 |
| 218145_at | NM_021158.1 | Putative protein kinase (C20orf97)† | 5.2 | 0.1 | 316 |
| 205830_at | NM_004362.1 | Calmegin (CLGN)†† | 5.0 | 1.1 | 63 |
| 218755_at | NM_005733.1 | Kinesin family member 20A (KIF20A)†† | −24.6 | 3.8 | 103 |
| 221521_s_at | BC003186.1 | DNA replication complex GINS protein PFS2 (PFS2) | −15.0 | 6.5 | 102 |
| 202095_s_at | NM_001168.1 | Baculoviral IAP repeat-containing 5 (survivin) (BIRC5)† | −14.7 | 9.5 | 151 |
| 204582_s_at | NM_001648.1 | Kallikrein 3 (prostate specific antigen) (KLK3) | −14.3 | 5.2 | 1139 |
| 206422_at | NM_002054.1 | Glucagon (GCG) | −13.7 | 6.9 | 103 |
| 203213_at | AL524035 | Cell division cycle 2 (CDC2)† | −13.6 | 0.4 | 366 |
| 204641_at | NM_002497.1 | NIMA (never in mitosis gene a)-related kinase 2 (NEK2) | −12.2 | 4.5 | 138 |
| 207245_at | NM_001077.1 | UDP glycosyltransferase 2 family, polypeptide B17 (UGT2B17) | −11.9 | 1.3 | 178 |
| 210052_s_at | AF098158.1 | TPX2, microtubule-associated protein homologue (Xenopus laevis) (TPX2)† | −11.1 | 6.6 | 195 |
| 204170_s_at | NM_001827.1 | CDC28 protein kinase regulatory subunit 2 (CKS2) | −10.5 | 0.4 | 544 |
| 204026_s_at | NM_007057.1 | ZW10 interactor (ZWINT)† | −9.9 | 0.8 | 422 |
| 218039_at | NM_016359.1 | Nucleolar protein ANKT (ANKT)† | −9.8 | 3.7 | 340 |
| 202870_s_at | NM_001255.1 | CDC20 (cell division cycle 20, S. cerevisiae, homologue) (CDC20) | −9.6 | 2.2 | 130 |
| 219148_at | NM_018492.1 | T-LAK cell originated protein kinase (TOPK)† | −9.3 | 0.1 | 211 |
| 203908_at | NM_003759.1 | Solute carrier family 4, member 4 (SLC4A4) | −8.6 | 3.1 | 393 |
| 203276_at | NM_005573.1 | Lamin B1 (LMNB1)† | −8.3 | 2.1 | 116 |
| 203755_at | NM_001211.2 | Budding uninhibited by benzimidazoles 1 (yeast homologue), beta (BUB1B)† | −8.0 | 1.2 | 169 |
| 202483_s_at | NM_002882.2 | RAN binding protein 1 (RANBP1) | −7.3 | 4.1 | 584 |
| 202107_s_at | NM_004526.1 | Minichromosome maintenance deficient (S. cerevisiae) 2 (MCM2) | −7.1 | 0.5 | 273 |
| 209773_s_at | BC001886.1 | Ribonucleotide reductase M2 polypeptide (RRM2)†† | −7.0 | 0.1 | 234 |
| 203362_s_at | NM_002358.2 | MAD2 (mitotic arrest deficient, yeast, homologue)-like 1 (MAD2L1) | −6.8 | 0.4 | 198 |
| 209854_s_at | AA595465 | Kallikrein 2, prostatic (KLK2) | −6.5 | 0.6 | 162 |
| 205433_at | NM_000055.1 | Butyrylcholinesterase (BCHE) | −6.1 | 3.1 | 356 |
| 222077_s_at | AU153848 | Rac GTPase activating protein 1 (RACGAP1)† | −6.1 | 0.2 | 157 |
| 209706_at | AF247704.1 | NK3 transcription factor related, locus 1 (Drosophila) (NKX3-1) | −5.9 | 2.9 | 828 |
| 218009_s_at | NM_003981.1 | Protein regulator of cytokinesis 1 (PRC1)† | −5.7 | 0.4 | 185 |
| 205345_at | NM_000465.1 | BRCA1 associated RING domain 1 (BARD1) | −5.7 | 1 | 291 |
| 212298_at | BE620457 | Neuropilin 1 (NRP1) | −5.6 | 1.8 | 160 |
| 204393_s_at | NM_001099.2 | Acid phosphatase, prostate (ACPP) | −5.4 | 0.3 | 299 |
| 209426_s_at | AF047020.1 | Alpha-methylacyl-CoA racemase (AMACR) | −5.4 | 2.2 | 137 |
| 202338_at | NM_003258.1 | Thymidine kinase 1, soluble (TK1)† | −5.0 | 0.2 | 170 |

[a]Fold ± Int represents mean fold change with interval that determines the primary values. [b]Ctrl refers to mean control expression level (fluorescence signal) and control samples marked [x] were called absent by absolute analysis algorithm. Genes marked † or †† were similarly changed in DU145 cells. Genes marked †† are also displayed in Table 3.

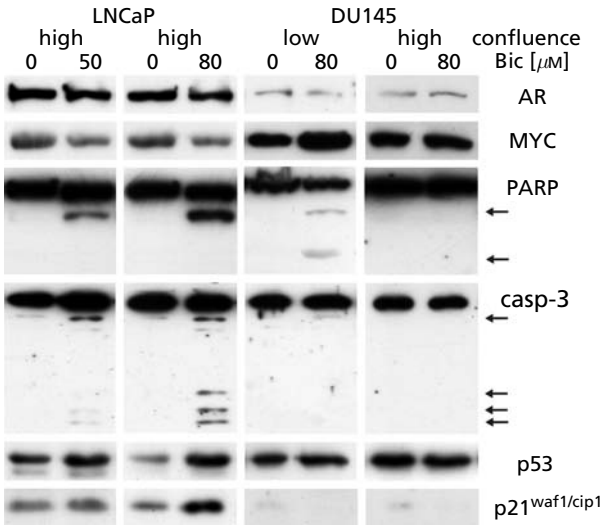


**Figure 1** Immunoblotting of selected proteins after 1 day of bicalutamide treatment (confluence low ~ 30–50% and high ~ 80–100%). Arrows indicate cleavage fragments of PARP (~ 60 and 89 kDa) and procaspase-3 (~ 30, 22, 19 and 17 kDa).

cells. Telomerase was not inhibited in LNCaP cells after 1 day of treatment and in DU145 cells after 1 or 3 days of treatment (Figure 3). The list of telomerase-related gene responses after bicalutamide treatment is presented in Table 2 (see Materials and Methods for design of microarray experiments). Transcripts for TERT were not detected in any sample, which was not in accordance with high telomerase activity in both cell lines. Since TERT transcripts could be rapidly modified with loss of the poly(A) tail, we analysed the TERT expression not only by microarray analysis where T7-(dT)24 primer against poly(A) tail is involved but also by real-time PCR, where the reverse transcription is based on specific primers. We confirmed the expression of TERT by real-time PCR in both cell lines (normalized TERT values were 0.050 for LNCaP cells and 0.041 for DU145 cells). Moreover, in LNCaP cells we found a decrease of TERT after both 1 day (8.5 and 5.6 fold) and 3 days (29.4 fold) of bicalutamide treatment. The expression of TERT was not changed in DU145 cells. The decrease of telomerase activity in LNCaP cells was also accompanied by a decrease in the mRNA level of MYC, androgen receptor, dyskerin and chaperone proteins HSP90, p23 and Hsp70Hsp90-organizing protein (Table 2). Immunoblotting of MYC and androgen receptor confirmed their decrease in protein level (Figure 1). Recently reported repressors of TERT promotor, menin, SIP1 and MAD1 were not changed in any cell line and treatment (data not shown). Stimulation of LNCaP cells by dihydrotestosterone, as well as by 17$\beta$-estradiol, was associated with slightly increased mRNA levels of MYC (both 1.4-fold;

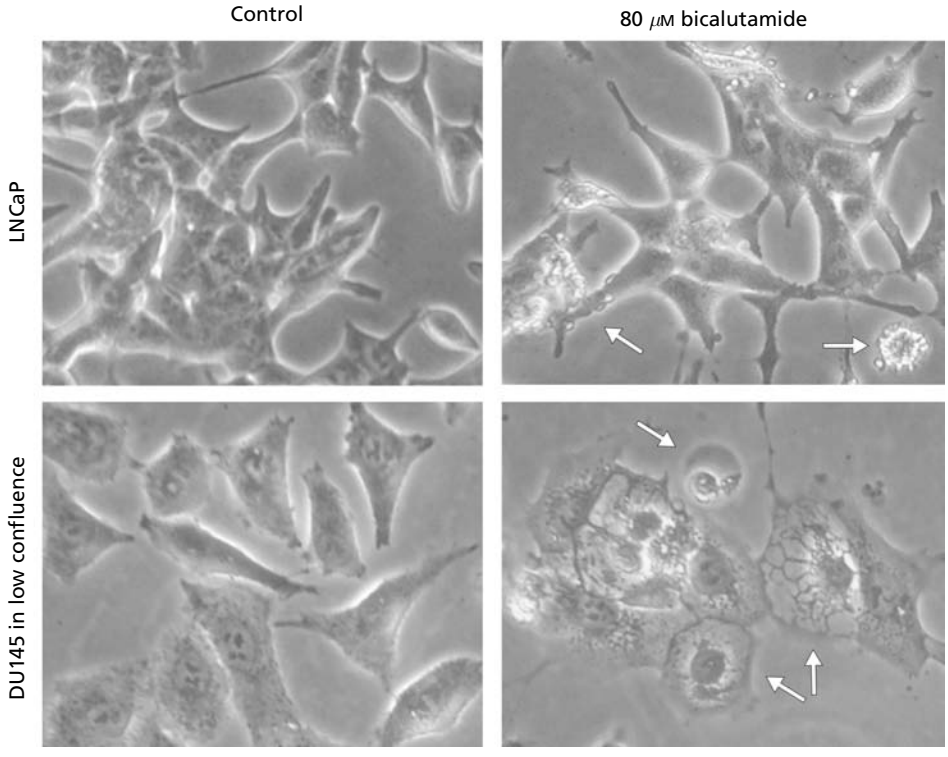


**Figure 2** Morphology of LNCaP and DU145 cells after 1 day of treatment with 80 $\mu$M bicalutamide. Arrows indicate membrane blebbing and cellular fragmentation of LNCaP cells and vacuolization, clearance of cytoplasm and formation of large bubbles in DU145 cells Magnification, 200× by light microscope.

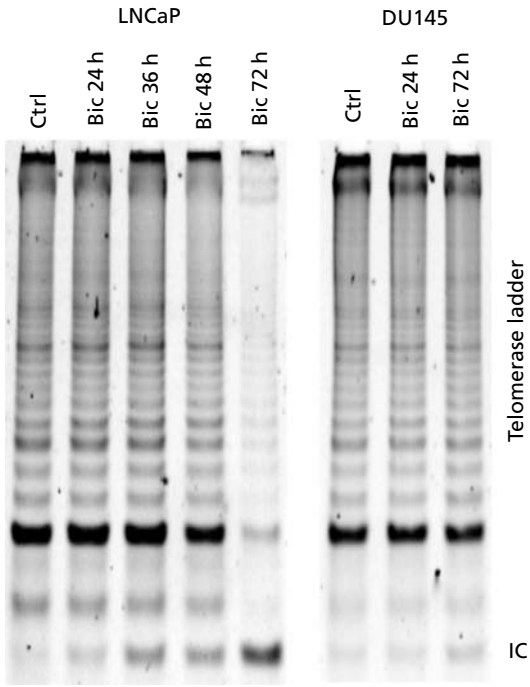


**Figure 3** Telomerase activity in LNCaP and DU145 cells treated by 80 $\mu$M bicalutamide for different time intervals (IC, internal control for PCR amplification). Telomerase activity in control samples did not change over time.

data from microarray analysis) and TERT (1.7- and 2.1-fold, respectively; data from real-time PCR) but not with increase of MYC protein and telomerase activity (data not shown).

### Influence of cell confluence on effect of bicalutamide in androgen-insensitive DU145 cells

We measured a similar $IC_{50}$ for bicalutamide both in androgen-sensitive LNCaP (40 $\mu$M) and insensitive DU145 (56.7 $\mu$M) cell lines when growing in low confluence. Although DU145 cells were significantly more resistant in several concentrations (Figure 4), explanation of the androgen receptor independent effects of bicalutamide together with its specific inhibition of telomerase in LNCaP cells was the primary aim of this microarray analysis. In order to get sufficient mRNA we cultivated cells in higher confluence (approximately 80%). As has already been stated, the number of genes significantly altered after bicalutamide treatment in DU145 cells was more than three times lower than in androgen-sensitive LNCaP cells. Reanalysis of the $IC_{50}$ of bicalutamide in high cell confluence (Figure 4) showed the survival of more than 50% of DU145 cells even at 150 $\mu$M. Treatment of DU145 cells growing in low confluence (up to 60%) by 80 $\mu$M bicalutamide resulted in the formation of large bubbles on the cell surface, clearance of the cytoplasm and vacuolization (Figure 2). This was associated with upregulation of MYC and cleavage of PARP protein into two fragments of approximately 89 and 60 kDa, but not with cleavage of procaspase-3 (Figure 1). PARP cleavage and upregulation of MYC after bicalutamide treatment was not observed in DU145 cells growing in high confluence and also incidence of the mentioned morphological changes was lower. Downregulation of protein $p21^{waf1/cip1}$ was observed in cells grown at both low and high confluence. Microarray analysis of DU145 cells growing in high confluence revealed that more than 50% of increased/decreased genes were also changed in the LNCaP androgen-sensitive cell line (87/89 genes in both

**Table 2** Telomerase-related genes after treatment with 80 $\mu$M bicalutamide

| Affymetrix ID | GenBank ID | Description | LNCaP | | | | DU145 | |
|---|---|---|---|---|---|---|---|---|
| | | | 1 day | | 3 days | | 1 day | |
| | | | Fold[a] | Ctrl[b] | Fold | Ctrl | Fold | Ctrl |
| 207199_at | NM_003219.1 | Telomerase reverse transcriptase (TERT) | NC[c] | 5[x] | NC | 7[x] | NC | 9[x] |
| 202431_s_at | NM_002467.1 | v-myc avian myelocytomatosis viral oncogene homologue (MYC) | −4.5 | 364 | −10.1 | 209 | 1.5[nc] | 248 |
| 211110_s_at | AF162704.1 | Androgen receptor (AR) | −3.1 | 210 | −2.7 | 255 | NC | 42[x] |
| 201479_at | NM_001363.1 | Dyskerin (DKC1) | −2.4 | 431 | −1.6 | 332 | NC | 366 |
| 213330_s_at | BE886580 | Hsp70Hsp90-organizing protein (HOP) | −2.1 | 186 | NC | 100 | NC | 216 |
| 200064_at | AF275719.1 | Chaperone protein HSP90 beta (HSPCB) | −1.6 | 1683 | −1.3 | 1317 | NC | 1770 |
| 211969_at | BG420237 | Chaperone protein HSP90 alpha (HSPCA) | −1.6 | 1251 | NC | 983 | −1.5[nc] | 1249 |
| 200627_at | BC003005.1 | Telomerase-binding protein p23 (TEBP) | −1.5 | 1072 | −1.5 | 776 | −1.4[nc] | 1108 |

[a]Fold represents fold change of gene expression. Data for 1 day of treatment of LNCaP cells represent mean fold change. [b]Ctrl refers to expression level (fluorescence signal) of single control or mean value. Control samples marked [x] were called absent by absolute analysis algorithm. [c]NC indicates that the second sample was not changed. Expression of TERT was reanalysed by real-time RT-PCR (see text). Probe set for RNA subunit of telomerase was not included on the Human Genome Focus Array.

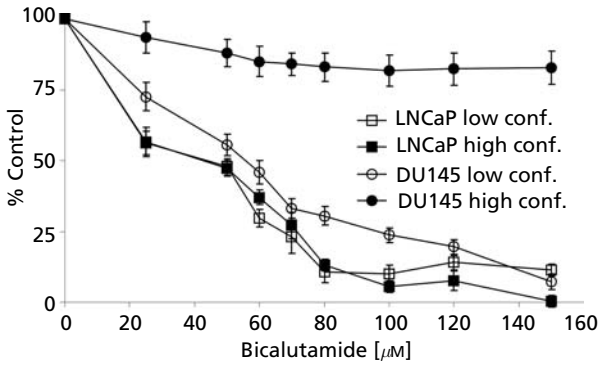


**Figure 4** Inhibition of cell viability by 3-day bicalutamide treatment of LNCaP and DU145 cells with respect to cell confluence (low ~ 30–50%, high ~ 80–100%). Data represent mean values with standard deviations from three independent experiments. DU145 cells grown in high confluence were significantly more resistant in all concentrations tested in comparison to DU145 cells grown in low confluence, as well as to LNCaP cells grown in both confluences ($P < 0.001$). DU145 cells grown in low confluence were significantly more resistant than LNCaP cells grown in low (25, 60, 80 and 100 $\mu$M) and high (25, 80, 100 and 120 $\mu$M) confluences in indicated concentrations ($P < 0.05$). A significant difference between LNCaP cells grown in low and high confluences was detected for a concentration of 150 $\mu$M of bicalutamide ($P < 0.05$).

cell lines). The genes with the highest change in both cell lines are marked in Tables 1 and 3.

## Discussion

The LNCaP cell line contains wild type p53, carries a mutation in the hormone-binding domain of androgen receptor and expresses moderate levels of this receptor (Veldscholte et al 1992; Carroll et al 1993). Androgen-insensitive DU145 cells have a mutated p53 gene and expression of androgen receptor is inhibited by promotor methylation (Carroll et al 1993; Jarrard et al 1998). The importance of androgen receptor and factors regulating its activity and expression in prostate cancer was recently reviewed by Culig et al (2003). Chen et al (2004) reported a novel finding, that the increased level of the functional androgen receptor is probably the major change associated with progression to hormone refractory prostate cancer.

Inhibition of telomerase activity after 3 days of bicalutamide treatment in LNCaP cells was accompanied with the downregulation of TERT, MYC and androgen receptor. This accords with the role of MYC as a TERT transcription factor (Oh et al 1999) and with the conventional

**Table 3** Genes changed at least three-fold after 1 day of treatment with 80 $\mu$M bicalutamide in DU145 cells

| Affymetrix ID | GenBank ID | Description | Fold ± Int[a] | | Ctrl[b] |
|---|---|---|---|---|---|
| 212531_at | NM_005564.1 | Lipocalin 2 (oncogene 24p3) (LCN2) | 7.6 | 0.5 | 33[x] |
| 207850_at | NM_002090.1 | GRO3 oncogene (GRO3) | 5.2 | 0.2 | 15[x] |
| 200924_s_at | NM_002394.1 | Solute carrier family, member 2 (SLC3A2)†† | 4.1 | 0.7 | 179 |
| 219117_s_at | NM_016594.1 | FK506 binding protein precursor (LOC51303)† | 4.1 | 0.2 | 129 |
| 203234_at | NM_003364.1 | Uridine phosphorylase (UP)†† | 4.0 | 0.4 | 594 |
| 209035_at | M69148.1 | Midkine (neurite growth-promoting factor 2) (MDK) | 3.9 | 0.9 | 30[x] |
| 200825_s_at | NM_006389.2 | Oxygen-regulated protein (150 kD) (ORP150)† | 3.7 | 0.3 | 398 |
| 205830_at | NM_004362.1 | Calmegin (CLGN)†† | 3.7 | 0.1 | 40 |
| 202842_s_at | AL080081.1 | DnaJ (Hsp40) homologue, subfamily B, member 9 (DNAJB9)†† | 3.6 | 0.1 | 123 |
| 217967_s_at | AF288391.1 | Niban protein (C1orf24)†† | 3.2 | 0.3 | 229 |
| 200629_at | NM_004184.2 | Tryptophanyl-tRNA synthetase (WARS)† | 3.2 | 0.4 | 200 |
| 217988_at | NM_021178.1 | Chromosome 14 open reading frame 18 (C14orf18) | 3.1 | 0.3 | 153 |
| 205569_at | NM_014398.1 | Lysosome-associated membrane glycoprotein (LAMP3)† | 3.1 | 0.4 | 61 |
| 209773_s_at | BC001886.1 | Ribonucleotide reductase M2 polypeptide (RRM2)†† | −5.3 | 0.1 | 958 |
| 204159_at | NM_001262.1 | Cyclin-dependent kinase inhibitor 2C (p18, INK4C) (CDKN2C)† | −4.5 | 1.4 | 102 |
| 203153_at | NM_001548.1 | Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) | −3.8 | 0.2 | 194 |
| 203108_at | NM_003979.2 | Retinoic acid induced 3 (RAI3) | −3.7 | 0.1 | 711 |
| 218755_at | NM_005733.1 | Kinesin family member 20A (KIF20A)†† | −3.5 | 0.4 | 300 |
| 202756_s_at | NM_002081.1 | Glypican 1 (GPC1) | −3.2 | 0.4 | 215 |
| 201202_at | NM_002592.1 | Proliferating cell nuclear antigen (PCNA)† | −3.2 | 0.7 | 226 |
| 202735_at | NM_006579.1 | Emopamil-binding protein (sterol isomerase) (EBP)† | −3.3 | 1.1 | 148 |
| 212020_s_at | AU152107 | Antigen identified by monoclonal antibody Ki-67 (MKI67) | −3.0 | 0.2 | 144 |

[a]Fold ± Int represents mean fold change with interval that determines the primary values. [b]Ctrl refers to control expression level (fluorescence signal) and control samples marked [x] were called absent by absolute analysis algorithm. Genes marked † or †† were similarly changed also in LNCaP cells. Genes marked †† are also displayed in Table 1.

correlation of the expression of the catalytic subunit of telomerase with telomerase activity (Collins & Mitchell 2002). MYC was shown to be upregulated by androgens in prostatic epithelial cells (Silva et al 2001) and MYC family proteins are implicated in autoregulation of the androgen receptor (Grad et al 1999). One-day stimulation of LNCaP cells by dihydrotestosterone and 17$\beta$-estradiol was associated with increased mRNA levels of MYC and TERT in LNCaP cells but not with increased telomerase activity. This could be related to the short treatment interval. The stimulation of telomerase by androgens was reported by Soda et al (2000) and Guo et al (2003). Guo and colleagues also proved that androgens regulate TERT indirectly, which is in accordance with the pathway discussed above. Androgen receptor specific regulation of TERT is further supported by no effect of bicalutamide on telomerase activity in the androgen-insensitive cell line DU145. Downregulation of telomerase activity in LNCaP cells could be further affected by decreased mRNA levels of dyskerin and HSP90 proteins. Dyskerin stabilizes the RNA subunit of telomerase (Collins & Mitchell 2002) and the chaperone HSP90 complex is important for telomerase assembly (Akalin et al 2001). It has been recently reported that complete androgen ablation by bicalutamide and goserelin acetate resulted in a decrease of TERT protein (Iczkowski et al 2004). However, inhibition of telomerase probably does not lead to telomere erosion and replicative senescence because androgen ablation is associated with growth arrest of tumour cells (Guo et al 2003).

One of the major responses of LNCaP cells after treatment with 80 $\mu$M bicalutamide was activation of p53 protein and its downstream regulated genes. Activation of p53 is probably not due to DNA damage because in-vitro as well as in-vivo genotoxic tests were negative even in higher concentrations (Iswaran et al 1997). Bicalutamide treatment was also associated with apoptotic cleavage of procaspase-3 and PARP. Our results are in good agreement with Lee et al (2003), who described the activation of caspase-3 and caspase-8, associated with translocation and cleavage of BAX after 2-day treatment of LNCaP cells with 100 $\mu$M bicalutamide. Treatment of LNCaP cells with 50 $\mu$M bicalutamide, which is comparable to the mean plasma concentration of 150 mg daily in monotherapy (Tyrrell et al 1998), resulted in a weaker response than after treatment with a concentration of 80 $\mu$M. Slight changes in p53 and p21 protein levels after treatment with 50 $\mu$M bicalutamide were unfortunately misinterpreted in our previous work (Bouchal et al 2002). Higher dosages of bicalutamide are not used in clinical practice because of a plateau in the PSA response beyond 200 mg daily (Kolvenbag et al 1998). With respect to the good tolerability of bicalutamide in dosages up to 600 mg daily and the stronger response to the 80 $\mu$M dose of bicalutamide described above, the treatment with higher dosages could have clinical importance.

Neither the telomerase activity nor the p53 pathway were affected in the androgen-insensitive cell line DU145 but when growing in low confluence the viability was inhibited at a similar concentration of bicalutamide as in androgen-sensitive LNCaP cells. High confluent DU145 cells were more resistant to bicalutamide; this is in good accordance with the observed low clinical effect of bicalutamide against refractory prostate carcinomas (Balk 2002; Oh 2002). The inhibition effects seen with bicalutamide specifically in low cell confluence were reported also for TGF$\beta$1 (Morton & Barrack 1995). The action of TGF$\beta$1 on poorly differentiated rat prostate cancer cells was further affected by serum, growth factors and extracellular matrix, which could also be important effects in the case of bicalutamide. Furthermore, DU145 cells are grown in monolayers and a high confluence could result in retardation of the cell cycle and, subsequently, in a lower sensitivity to bicalutamide. Our in-vitro test revealed that more than 50% of genes changed after bicalutamide treatment of high confluent DU145 cells were also changed in androgen-sensitive LNCaP cells. The amplitude of the change could be higher in DU145 cells growing in low confluence, which exhibited PARP cleavage into fragments of 89 kDa and approximately 60 kDa but not apoptotic cleavage of procaspase-3. Cleavage of PARP into a 60 kDa fragment after $\beta$-lapachone treatment was reported by Planchon et al (2001), who explained this by activation of calpains. Bicalutamide treatment was further accompanied by an increase in MYC and a decrease in $p21^{waf1/cip1}$ proteins. These changes were reported to be associated with apoptosis in a number of systems (Dang 1999; Gartel & Tyner 2002) but the morphological changes of DU145 cells do not support this type of cell death. The major morphologic changes after the treatment were the formation of large bubbles on the cell surface and vacuolization of the cytoplasm, which could be indicators of necrosis and autophagic degeneration, respectively (Ogier-Denis & Codogno 2003). Further work is needed to define the types of cell death observed after treatment with bicalutamide as well as to elucidate biological relationships between the genes defined by the microarray analysis. So far, we can only speculate about the meaning of the observed changes in gene expression. In DU145 cells the highest upregulation after bicalutamide treatment was observed for lipocalin 2, which was reported to be one of the acute phase response genes (Liu & Nilsen-Hamilton 1995). It could be induced by various stressful stimuli and probably plays an anti-inflammatory role. In rodents, it was reported to be upregulated by serum, basic fibroblast growth factor and phorbol esters (Liu & Nilsen-Hamilton 1995), items that could induce also deiodinase 3 (Hernandez & St Germain 2002), the most highly upregulated gene in response to bicalutamide in LNCaP cells. Bicalutamide treatment may cause cellular stress, which could also explain the activation of the p53 pathway in LNCaP cells. In this cell line the most highly downregulated gene was kinesin family member 20A, which is engaged in intracellular transport and cell division (Hill et al 2000). Ribonucleotide reductase M2, which is involved in DNA synthesis (Zhou & Yen 2001), decreased the most in DU145 cells. Importantly, these two genes were also highly downregulated in the other cell line, which could provide clues to the similar response to bicalutamide in both LNCaP and DU145 cells. We hope that our in-vitro observations will be validated in-vivo, as in case of telomerase inhibition (Bouchal et al 2002; Iczkowski et al 2004).

## Conclusions

We describe the MYC protein as the missing link in the regulation of telomerase reverse transcriptase by the androgen receptor. Downregulation of telomerase activity by bicalutamide probably does not have any clinical impact because it is associated with the inhibition of proliferation and therefore telomerase erosion and the induction of senescence could not occur. Another new finding, which could have clinical importance, is the activation of the p53 pathway after treatment with 80 $\mu$M bicalutamide. Activation of the p53 pathway and the induction of apoptosis were also observed to a lesser extent after treatment with 50 $\mu$M bicalutamide, which is equivalent to 150 mg daily. Higher doses of bicalutamide are well tolerated and therefore their usage could potentially bring benefit to patients with androgen-sensitive carcinomas. Finally, we observed an androgen receptor independent effect of the anti-androgen bicalutamide on DU145 cells grown at a low confluence. Androgen-insensitive DU145 cells grown at a high confluence exhibited a higher resistance to bicalutamide, which is in accordance with the ineffectiveness of bicalutamide in the treatment of androgen-insensitive prostate carcinomas. In-vitro experiments with low confluent DU145 cells therefore have major limitations. According to microarray analysis a subset of genes was similarly changed in both the LNCaP and DU145 cells, which suggests additional effects of the anti-androgen bicalutamide. We have provided the microarray data for further verification and biovalidation, and we hope this will contribute to a better understanding of androgen receptor signalling and bicalutamide action.

## References


Akalin, A., Elmore, L. W., Forsythe, H. L., Amaker, B. A., McCollum, E. D., Nelson, P. S., Ware, J. L., Holt, S. E. (2001) A novel mechanism for chaperone-mediated telomerase regulation during prostate cancer progression. *Cancer Res.* **61**: 4791–4796

Balk, S. P. (2002) Androgen receptor as a target in androgen-independent prostate cancer. *Urology* **60** (suppl. 3A): 132–139

Blackburn, E. H. (1992) Telomerases. *Annu. Rev. Biochem.* **61**: 113–129

Bouchal, J., Kolar, Z., Mad'arova, J., Hlobilkova, A., von Angerer, E. (2002) The effects of natural ligands of hormone receptors and their antagonists on telomerase activity in the androgen sensitive prostatic cancer cell line LNCaP. *Biochem. Pharmacol.* **63**: 1177–1181

Carroll, A. G., Voeller, H. J., Sugars, L., Gelmann, E. P. (1993) p53 oncogene mutations in three human prostate cancer cell lines. *Prostate* **23**: 123–134

Chen, C. D., Welsbie, D. S., Tran, C., Baek, S. H., Chen, R., Vessella, R., Rosenfeld, M. G., Sawyers, C. L. (2004) Molecular determinants of resistance to antiandrogen therapy. *Nat. Med.* **10**: 33–39

Cockshott, I. D., Cooper, K. J., Sweetmore, D. S., Blacklock, N. J., Denis, L. (1990) The pharmacokinetics of Casodex in prostate cancer patients after single and during multiple dosing. *Eur. Urol.* **18** (suppl. 3): 10–17

Collins, K., Mitchell, J. R. (2002) Telomerase in the human organism. *Oncogene* **21**: 564–579

Culig, Z., Klocker, H., Bartsch, G., Steiner, H., Hobisch, A. (2003) Androgen receptors in prostate cancer. *J. Urol.* **170**: 1363–1369

Dang, C. V. (1999) c-Myc target genes involved in cell growth, apoptosis, and metabolism. *Mol. Cell Biol.* **19**: 1–11

Denis, L., Murphy, G. P. (1993) Overview of phase III trials on combined androgen treatment in patients with metastatic prostate cancer. *Cancer* **72**: 3888–3895

Furr, B. J., Valcaccia, B., Curry, B., Woodburn, J. R., Chesterson, G., Tucker, H. (1987) ICI 176,334: a novel non-steroidal, peripherally selective antiandrogen. *J. Endocrinol.* **113**: R7–9

Gartel, A. L., Tyner, A. L. (2002) The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Mol. Cancer Ther.* **1**: 639–649

Grad, J. M., Dai, J. L., Wu, S., Burnstein, K. L. (1999) Multiple androgen response elements and a Myc consensus site in the androgen receptor (AR) coding region are involved in androgen-mediated up-regulation of AR messenger RNA. *Mol. Endocrinol.* **13**: 1896–1911

Guo, C., Armbruster, B. N., Price, D. T., Counter, C. M. (2003) In vivo regulation of hTERT expression and telomerase activity by androgen. *J. Urol.* **170**: 615–618

Harley, C. B. (1991) Telomere loss: mitotic clock or genetic time bomb? *Mutat. Res.* **256**: 271–282

Hernandez, A., St Germain, D. L. (2002) Dexamethasone inhibits growth factor-induced type 3 deiodinase activity and mRNA expression in a cultured cell line derived from rat neonatal brown fat vascular-stromal cells. *Endocrinology* **143**: 2652–2658

Hill, E., Clarke, M., Barr, F. A. (2000) The Rab6-binding kinesin, Rab6-KIFL, is required for cytokinesis. *EMBO J.* **19**: 5711–5719

Iczkowski, K. A., Huang, W., Mazzucchelli, R., Pantazis, C. G., Stevens, G. R., Montironi, R. (2004) Androgen ablation therapy for prostate carcinoma suppresses the immunoreactive telomerase subunit hTERT. *Cancer* **100**: 294–299

Iswaran, T. J., Imai, M., Betton, G. R., Siddall, R. A. (1997) An overview of animal toxicology studies with bicalutamide (ICI 176,334). *J. Toxicol. Sci.* **22**: 75–88

Jarrard, D. F., Kinoshita, H., Shi, Y., Sandefur, C., Hoff, D., Meisner, L. F., Chang, C., Herman, J. G., Isaacs, W. B., Nassif, N. (1998) Methylation of the androgen receptor promoter CpG island is associated with loss of androgen receptor expression in prostate cancer cells. *Cancer Res.* **58**: 5310–5314

Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L., Shay, J. W. (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**: 2011–2015

Kolvenbag, G. J., Nash, A. (1999) Bicalutamide dosages used in the treatment of prostate cancer. *Prostate* **39**: 47–53

Kolvenbag, G. J., Blackledge, G. R., Gotting-Smith, K. (1998) Bicalutamide (Casodex) in the treatment of prostate cancer: history of clinical development. *Prostate* **34**: 61–72

Lee, E. C., Zhan, P., Schallhom, R., Packman, K., Tenniswood, M. (2003) Antiandrogen-induced cell death in LNCaP human prostate cancer cells. *Cell Death Differ.* **10**: 761–771

Liu, Q., Nilsen-Hamilton, M. (1995) Identification of a new acute phase protein. *J. Biol. Chem.* **270**: 22565–22570

Morton, D. M., Barrack, E. R. (1995) Modulation of transforming growth factor beta 1 effects on prostate cancer cell proliferation by growth factors and extracellular matrix. *Cancer Res.* **55**: 2596–2602

Ogier-Denis, E., Codogno, P. (2003) Autophagy: a barrier or an adaptive response to cancer. *Biochim. Biophys. Acta* **1603**: 113–128

Oh, W. K. (2002) Secondary hormonal therapies in the treatment of prostate cancer. *Urology* **60** (suppl. 3A): 87–93

Oh, S., Song, Y. H., Kim, U. J., Yim, J., Kim, T. K. (1999) In vivo and in vitro analyses of Myc for differential promoter activities of the human telomerase (hTERT) gene in normal and tumor cells. *Biochem. Biophys. Res. Commun.* **263**: 361–365

Planchon, S. M., Pink, J. J., Tagliarino, C., Bornmann, W. G., Varnes, M. E., Boothman, D. A. (2001) beta-Lapachone-induced apoptosis in human prostate cancer cells: involvement of NQO1/xip3. *Exp. Cell Res.* **267**: 95–106

Romijn, J. C., Verkoelen, C. F., Schroeder, F. H. (1988) Application of the MTT assay to human prostate cancer cell lines in vitro: establishment of test conditions and assessment of hormone-stimulated growth and drug-induced cytostatic and cytotoxic effects. *Prostate* **12**: 99–110

Sasik, R., Calvo, E., Corbeil, J. (2002) Statistical analysis of high-density oligonucleotide arrays: a multiplicative noise model. *Bioinformatics* **18**: 1633–1640

Schellhammer, P. F. (2002) An evaluation of bicalutamide in the treatment of prostate cancer. *Expert Opin. Pharmacother.* **3**: 1313–1328

Silva, I. S., Morsch, D. M., Urnauer, L., Spritzer, P. M. (2001) Androgen-induced cell growth and c-myc expression in human non-transformed epithelial prostatic cells in primary culture. *Endocr. Res.* **27**: 153–169

Soda, H., Raymond, E., Sharma, S., Lawrence, R., Davidson, K., Oka, M., Kohno, S., Izbicka, E., Von Hoff, D. D. (2000) Effects of androgens on telomerase activity in normal and malignant prostate cells in vitro. *Prostate* **43**: 161–168

Toda, K., Ishida, S., Nakata, K., Matsuda, R., Shigemoto-Mogami, Y., Fujishita, K., Ozawa, S., Sawada, J., Inoue, K., Shudo, K., Hayashi, Y. (2003) Test of significant differences with a priori probability in microarray experiments. *Anal. Sci.* **19**: 1529–1535

Tyrrell, C. J., Denis, L., Newling, D., Soloway, M., Channer, K., Cockshott, I. D. (1998) Casodex 10–200 mg daily, used as monotherapy for the treatment of patients with advanced prostate cancer. An overview of the efficacy, tolerability and pharmacokinetics from three phase II dose-ranging studies. Casodex Study Group. *Eur. Urol.* **33**: 39–53

Veldscholte, J., Berrevoets, C. A., Ris-Stalpers, C., Kuiper, G. G., Jenster, G., Trapman, J., Brinkmann, A. O., Mulder, E. (1992) The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. *J. Steroid. Biochem. Mol. Biol.* **41**: 665–669

Zhou, B., Yen, Y. (2001) Characterization of the human ribonucleotide reductase M2 subunit gene; genomic structure and promoter analyses. *Cytogenet. Cell Genet.* **95**: 52–59