

Inhibition of MCF-7 breast cancer cell proliferation by 5 α -dihydrotestosterone; a role for p21^{Cip1/Waf1}

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Abstract

Androgens inhibit the growth of breast cancer cells *in vitro* and *in vivo* by mechanisms that remain poorly defined. In this study, treatment of asynchronously growing MCF-7 breast cancer cells with the androgen, 5 α -dihydrotestosterone (DHT), was shown to inhibit cell proliferation and induce moderate increases in the proportion of G1 phase cells. Consistent with targeting the G1-S phase transition, DHT pretreatment of MCF-7 cultures impeded the serum-induced progression of G1-arrested cells into S phase and reduced the kinase activities of cyclin-dependent kinase (Cdk)4 and Cdk2 to less than 50% of controls within 3 days. DHT treatment was associated with greater than twofold increases in the levels of the Cdk inhibitor, p27^{Kip1}, while p21^{Cip1/Waf1} protein levels remained unchanged. During the first 24 h of DHT treatment, levels of Cdk4-associated p21^{Cip1/Waf1} and p27^{Kip1} were reduced coinciding with decreased levels of Cdk4-associated cyclin D3. In contrast, DHT treatment caused increased accumulation of Cdk2-associated p21^{Cip1/Waf1}, with no significant alterations in levels of p27^{Kip1} bound to Cdk2 complexes. These findings suggest that DHT reverses the Cdk4-mediated titration of p21^{Cip1/Waf1} and p27^{Kip1} away from Cdk2 complexes, and that the increased association of p21^{Cip1/Waf1} with Cdk2 complexes in part mediates the androgen-induced growth inhibition of breast cancer cells.

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Introduction

Breast cancer is a steroid hormone-responsive disease and tumours frequently co-express receptors for oestrogen (ER), progesterone and androgens (AR). The high frequency of AR expression in both primary breast tumours (70–90% (Lea *et al.* 1989, Soreide *et al.* 1992, Kimura *et al.* 1993)) and in breast tumour metastases (75% (Lea *et al.* 1989)) suggests that androgens are important regulators of breast cancer cell proliferation. Indeed, androgens and androgenic compounds, including testosterone propionate (Cooperative Breast Cancer Group CBC 1964) and fluoxymesterone (Ingle *et al.* 1991), inhibit breast tumour growth *in vivo* and demonstrate a therapeutic efficacy comparable with other hormonal therapies, such as anti-oestrogens (Tormey *et al.* 1983, Ingle *et al.* 1991).

In vitro, androgens induce divergent proliferative effects in breast cancer cell lines, with responses

dependent on the cell line used, culture conditions and the concentration of natural or synthetic androgens employed (Poulin *et al.* 1988, Hackenberg *et al.* 1991, Marugo *et al.* 1992, Birrell *et al.* 1995, Menjo *et al.* 1998, Lapointe & Labrie 2001). Although androgen-induced growth inhibition is associated with decreased ER expression (Poulin *et al.* 1989, Zhou *et al.* 2000), androgens have been shown to inhibit both basal and oestrogen-induced breast cancer cell proliferation (Poulin *et al.* 1988, Zhou *et al.* 2000) and their effects are additive to that induced by anti-oestrogens (Dauvois *et al.* 1991). These results indicate that androgens impede breast cancer cell proliferation by a mechanism in addition to reducing oestrogen responsiveness.

The non-aromatisable androgen, 5 α -dihydrotestosterone (DHT), inhibits the oestrogen-stimulated growth of ZR-75-1 (de Launoit *et al.* 1991) and CAMA-1 (Lapointe & Labrie 2001)

breast cancer cells and the serum-induced growth of MDA-MB-453 breast cancer cells (Yeap *et al.* 1999). Results within these studies indicate that DHT treatment resulted in gradual and progressive decreases in the proportion of cells in S phase with concomitant increases in G1 phase cells. The progression of normal cells through G1 and into S phase involves the co-ordinated phosphorylation of retinoblastoma (Rb) and other pocket proteins by cyclin-dependent kinase (Cdk)/cyclin complexes, and the subsequent release of E2F and DP transcription factors required for the expression of genes essential for S phase progression. Sufficient phosphorylation of Rb and the ensuing transit of cells through the G1/S phase transition is dependent on the accumulation of G1 cyclin proteins and their assembly into active Cdk/cyclin complexes (Sherr 1994). In addition to the regulation of Cdk and cyclin expression by mitogens during G1 phase, Cdk activity can also be regulated by the phosphorylation and dephosphorylation of specific, conserved residues (Poon & Hunter 1995, Morgan 1996) and by their interaction with inhibitors.

Two families of structurally and functionally distinct Cdk inhibitors (Cdkis) have been characterised to date. The INK4 proteins (p15^{INK4B}, p16^{INK4A}, p18^{INK4C}, p19^{INK4D}) are involved in the control of Cdk(4/6)/cyclin D complex activity, with studies suggesting an important role in governing the binding of D-type cyclins to Cdk4 (Parry *et al.* 1995) and the redistribution of Cip/Kip inhibitors between Cdk/cyclin complexes (Reynisdottir *et al.* 1995, Reynisdottir & Massague 1997, McConnell *et al.* 1999). Members of the Cip/Kip inhibitor family include p21^{Cip1/Waf1}, p27^{Kip1} and p57^{Kip2}, and although these proteins associate with a range of Cdk/cyclin complexes *in vitro*, they appear to bind with higher affinity to the G1 complexes, Cdk(4/6)/cyclin D and Cdk2/cyclin E (Blain *et al.* 1997). The inhibitory properties of these proteins stem from their ability to bind to the Cdk subunit of Cdk/cyclin complexes and prevent the binding of ATP necessary for activating phosphorylation (Aprelikova *et al.* 1995, Saha *et al.* 1997). However, the role of Cip/Kip proteins in proliferating cells is complex, since these proteins are also essential components of active Cdk(4/6)/cyclin D complexes (Cheng *et al.* 1999, Muraoka *et al.* 2001). This apparent paradox arises from a dual functionality *in vivo*, with Cip/Kip proteins acting as assembly

factors necessary for the association of D-type cyclins with Cdk4/6 at lower concentrations and as Cdk inhibitors at higher concentrations (LaBaer *et al.* 1997, Sherr & Roberts 1999). In addition, p21^{Cip1/Waf1} and p27^{Kip1} contain nuclear localisation signals responsible for the translocation of Cdk(4/6)/cyclin D complexes to the nucleus where the phosphorylation of pocket proteins can occur (LaBaer *et al.* 1997).

In vivo, p21^{Cip1/Waf1} and p27^{Kip1} are more potent inhibitors of Cdk2/cyclin E than Cdk(4/6)/cyclin D and are involved in the normal progression of cells through G1 phase (Polyak *et al.* 1994, Blain *et al.* 1997). Since both p21^{Cip1/Waf1} and p27^{Kip1} are implicated in the assembly of cyclin D with Cdk(4/6), it has been proposed that Cdk(4/6)/cyclin D complexes act to titrate these inhibitors away from Cdk2/cyclin E in late G1 when Cdk2 activity is essential for progression through the restriction point (Poon *et al.* 1995, Planas-Silva & Weinberg 1997, Perez-Roger *et al.* 1999). It follows, therefore, that modification of Cdk4, cyclin D and Cdk1 protein levels by mitogens can alter the distribution of Cip/Kip proteins between Cdk/cyclin complexes, and consequently affect Cdk4/cyclin D complex stability, Cdk2 activity and the transit of cells through the restriction point.

Expression of p27^{Kip1} is frequently downregulated in both breast (Catzavelos *et al.* 1997, Tan *et al.* 1997, Yang *et al.* 1998, Chu *et al.* 1999) and prostate (Guo *et al.* 1997, Fernandez *et al.* 1999) tumours and correlates with tumour aggression and poor prognosis. Elevated p27^{Kip1} levels and its redistribution to cyclin E/Cdk2 complexes have been implicated in both the growth inhibition of CAMA-1 human breast cancer cells by DHT (Lapointe & Labrie 2001) and the G1 arrest of LNCaP prostate cancer cell proliferation by high concentrations of androgens (Tsihlias *et al.* 2000, Hofman *et al.* 2001). p21^{Cip1/Waf1} is thought to be an important regulator of breast cancer cell proliferation (Prall *et al.* 1997), and is responsible for the inhibition of MCF-7 human breast cancer cell proliferation by anti-oestrogens (Skildum *et al.* 2001). Similarly, the relief of anti-oestrogen-induced G1 arrest of MCF-7 cells by oestrogens involves the redistribution of p21^{Cip1/Waf1} from Cdk2/cyclin E to Cdk4/cyclin D complexes (Planas-Silva & Weinberg 1997, Cariou *et al.* 2000, Carroll *et al.* 2000, Skildum *et al.* 2001). Although the gene encoding p21^{Cip1/Waf1} contains an

androgen response element within its promoter and its expression is upregulated by androgens in prostate cancer cells (Lu *et al.* 1999, 2000), the role of p21^{Cip1/Waf1} in the androgen regulation of breast and prostate cancer growth is not known. Similarly, the regulation of Cdk4 and D-type cyclin protein levels by androgens in human breast cancer cells and its effects on p21^{Cip1/Waf1} distribution have not been investigated.

In the current report, we investigate the mechanisms by which the androgen, DHT, inhibits the proliferation of MCF-7 breast cancer cells and present evidence that DHT targets the G1/S phase transition by a mechanism involving the downregulation of both Cdk4 and Cdk2 kinase activities following loss of p21^{Cip1/Waf1} from Cdk4 complexes and its increased association with Cdk2 complexes. This occurs without alterations in steady-state p21^{Cip1/Waf1} protein levels.

Materials and methods

Cell culture

MCF-7 human breast cancer cells obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), penicillin (100 IU/ml) and streptomycin (100 µg/ml). DHT (Sigma-Aldrich, Sydney, Australia) was dissolved in 100% ethanol and added to media immediately prior to use. For these studies, MCF-7 cultures were used in experiments up to 20 passages following thawing, experiments were repeated two to five times and representative blots are shown.

Proliferation studies and cell morphology

MCF-7 cells were passaged into six-well plates at a concentration of 1×10^4 cells/well. After 24 h, medium was replaced with RPMI 1640 containing 2% FCS and 10^{-10} to 10^{-7} M DHT (or vehicle). Triplicate wells were trypsinised and counted using a haemocytometer and results were analysed using Student's *t*-test. For investigation of morphological changes, cells were cultured in RPMI 1640 containing 2% FCS and either 10^{-8} M DHT or ethanol vehicle for 3 days prior to phase contrast microscopy.

Cell cycle analysis

To investigate the effects of DHT on cell cycle distribution, asynchronously growing MCF-7 cells were cultured in RPMI 1640 medium containing 2% FCS and either 10^{-8} M DHT or ethanol vehicle. Following trypsinisation, 1.5×10^6 cells were sequentially washed in serum-free RPMI 1640 medium and RPMI 1640 medium containing 5% dimethylsulphoxide, then incubated at 37 °C for 30 min with propidium iodide buffer (10 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 5% NP40, 50 µg/ml propidium iodide and 100 µg/ml RNase A). Cell cycle analysis was performed using a Beckman Coulter Epics XI-MCL flow cytometer (Beckman Coulter, Sydney, Australia). DNA content and cell cycle distribution were determined using Multiplus (Phoenix Flow Systems, San Diego, CA, USA).

To examine the effects of DHT on the G1/S phase transition, MCF-7 cells were pre-cultured for 72 h in RPMI 1640 medium containing 2% FCS and 10^{-8} M DHT or ethanol vehicle. Cells were then arrested in G1 by a further 24 h of culture in serum-free RPMI 1640 medium containing 10^{-8} M DHT (or vehicle) and cell cycle analysis was performed as described above at 24 h after the return of 2% FCS to the cultures.

Immunoblot analysis

Changes in the levels of cell cycle proteins were investigated in MCF-7 cells cultured in RPMI 1640 medium containing 2% FCS and 10^{-8} M DHT (or vehicle) for up to 10 days. Cell extracts were prepared as follows. Cells growing as subconfluent monolayers were washed twice with phosphate-buffered saline (PBS) and scraped into lysis buffer containing 50 mM Tris-HCl, pH 6.8, 10% sucrose, 2% SDS and 5% β-mercaptoethanol. Whole cell extracts (20 µl) were separated by SDS-PAGE (in 7.5%, 10% or 12% acrylamide gels) and transferred overnight to nitrocellulose membranes (Hybond-C; Amersham Pharmacia Biotech, Sydney, Australia). For immunoblot analysis, membranes were blocked with Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.4 and 150 mM NaCl) containing 3% dried milk powder for 1.5 h, then incubated with primary antibody (AR (M3562, 1:2000; DAKO, Sydney, Australia), cyclin A (H-432, 1:2000), cyclin B1 (GNS1, 1:250),

cyclin D1 (HD11, 1:100), cyclin E (M-20, 1:250), p130 (C-20, 1:500), β -actin (I-19, 1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin D3 (C28620, 1:500), Cdk2 (C18520, 1:2000), Cdk4 (68791A, 1:750), p21^{Cip1/Waf1} (C24420, 1:500), p27^{Kip1} (K25020, 1:2000; Transduction Laboratories, Becton Dickinson, Perth, Australia), cyclin E (14591A, 1:500), or Rb (14001A, 1:1000; PharMingen, Becton Dickinson, Perth, Australia) diluted in TBS containing 0.2% Tween 20 (TBST) and 1% dried milk powder for 1.5 h at room temperature. Blots were washed with TBST (3 \times 10 min), incubated for 1.5 h with horseradish peroxidase-conjugated secondary antibodies (anti-mouse (1:1000), anti-rabbit (1:2000); Silenus Laboratories, Melbourne, Australia) or anti-goat (sc-2020, 1:1000; Santa Cruz Biotechnology) diluted in TBST containing 1% dried milk powder then washed with TBST (3 \times 10 min). Immunoreactivity was visualised using enhanced chemiluminescence (Amersham Pharmacia Biotech) and densitometry analysis performed using Scion Image (Scion Corporation, Frederick, MD, USA) with protein levels standardised against β -actin blots.

Immunoprecipitations and immunodepletions

For the analysis of Cdk2 and Cdk4 protein complexes, cell monolayers were washed twice with ice-cold PBS and harvested into 1 ml modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, pH 7.4, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na₃VO₄, 1% NP40, 0.25% Na-deoxycholate and protease inhibitor cocktail (Roche Diagnostics Australia, Castle Hill, Australia)). Cell suspensions were mixed at 4 °C for 20 min to lyse cells and the lysates were centrifuged at 6000 *g* for 10 min at 4 °C. The resulting supernatants were pre-cleared with 100 μ l of a 50% protein G-sepharose (Amersham Pharmacia Biotech) slurry by mixing at 4 °C for 2 h. Aliquots (500 μ l) of pre-cleared supernatant were incubated with 6 or 8 μ l/ml anti-Cdk2 (68476E) or Cdk4 (68456E; PharMingen) polyclonal antibodies respectively, with mixing overnight at 4 °C. To precipitate the immunocomplexes, protein solutions were mixed gently with 100 μ l 50% protein G-sepharose for 2 h at 4 °C, and the beads captured by centrifugation for 20 s at 800 *g*. Supernatants were retained for immunodepletion analysis. Beads were washed three times with

ice-cold PBS, then pellets were resuspended in 60 μ l 2 \times sample buffer (250 mM Tris-HCl, pH 6.8, 10% glycerol, 2% β -mercaptoethanol, 0.8% SDS and 0.1% bromophenol blue) and boiled for 5 min. Supernatants (20 μ l) were electrophoresed in 14% SDS-PAGE gels and analysed by immunoblotting.

Cyclin-dependent kinase assays

For the investigation of Cdk2 and Cdk4 kinase activity, MCF-7 cells were harvested as described for immunoprecipitations above, except that modified RIPA buffer was prepared without Na-deoxycholate. For Cdk2 kinase activity, cell lysates were pre-cleared by mixing with 100 μ l 50% protein G-sepharose at 4 °C for 30 min. Aliquots of supernatant (200–300 μ l) were diluted to 400 μ l with RIPA buffer and incubated with 3 μ l anti-Cdk2 (68476E) for 2 h at 4 °C with mixing. Immunocomplexes were captured by the addition of 100 μ l 50% protein G-sepharose and incubating suspensions for 2 h at 4 °C. Beads were pelleted and washed four times with 300 μ l ice-cold PBS before adding 40 μ l cold reaction buffer (31.25 mM β -glycerophosphate, pH 7.2, 25 mM MOPS, 18.75 mM MgCl₂, 1.25 mM EGTA, 1.25 mM Na₃VO₄, 1.25 mM dithiothreitol, 25 μ M ATP and 10 μ M [γ -³²P]-ATP) with 20 μ g histone (H1) (Auspep; Upstate Biotechnology, Sydney, Australia) as the substrate. Kinase reactions were incubated for 30 min at 30 °C. For Cdk4 kinase activity, protein was precipitated as described above, except that lysates (700 μ l) were pre-cleared twice for 1 h with protein G-sepharose at 4 °C and 300 μ l aliquots of supernatant were incubated with 3 μ l of anti-Cdk4 (68456E) for 2 h at 4 °C with mixing. Kinase assays were performed as above by incubating beads at 30 °C for 15 min with 15 μ g of a fragment of pRb (amino acids 773–928) (Upstate Biotechnology) as the substrate. Following the termination of enzyme reactions by adding 50 μ l of 2 \times sample buffer to each tube, samples were boiled for 8 min and electrophoresed in 12% SDS-polyacrylamide gels. Phosphorylated bands were visualised by autoradiography following overnight exposure at –80 °C and quantitated using Scion Image. Immunoblots for Cdk2 and Cdk4 were used to confirm comparable immunoprecipitation of Cdk2 and Cdk4 respectively (results not shown).

Results

MCF-7 cells express the AR and are androgen responsive

The binding of androgens to the AR increases the stability of the protein, transiently elevating AR protein levels in a variety of cell types (Nemoto *et al.* 1986, Kempainen *et al.* 1992, Zhou *et al.* 1995). Few studies, however, have investigated the effects of androgens on AR protein levels in breast cancer cells. Yeap *et al.* (1999) reported increased AR protein levels without a corresponding increase in AR mRNA in DHT-treated MDA-MB-453 breast cancer cells. To confirm that MCF-7 cultures express the AR and to determine whether androgen treatment was associated with elevated AR protein levels, immunoblotting was performed using whole cell extracts prepared from cells cultured in the presence of 10^{-8} M DHT. MCF-7 cells continued to express the AR throughout a 10-day DHT treatment period with AR protein levels increased to 270% of controls by 2 days of treatment, and peaking at 320% of controls at 4 days (Fig. 1). Given that ligand binding to the AR has previously been shown to increase receptor stability (Krongrad *et al.* 1991, Zhou *et al.* 1995), these data suggest that MCF-7 is androgen responsive and that DHT induces a similar, transient increase in AR protein levels to that seen in other cell types.

DHT inhibits the proliferation of MCF-7 cells by targeting the G1/S phase transition

Conflicting studies have reported androgens to both induce (Birrell *et al.* 1995) and inhibit (de Launoit *et al.* 1991, Birrell *et al.* 1995, Lapointe & Labrie 2001, Ando *et al.* 2002, Ortmann *et al.* 2002) the proliferation of human breast cancer cell lines *in vitro*, with discrepancies in results due potentially to differences in culture conditions and different isolates of cell lines. The current study employed asynchronously growing MCF-7 cells to determine the effects of 10^{-10} to 10^{-7} M DHT on proliferation. As expected, cells growing in the absence of DHT proliferated steadily over 9 days of culture, with twofold increases in average cell number during the first and second 3-day intervals (Fig. 2A). By 9 days, the increase in cell number began to plateau as cells approached confluency. In contrast, treatment with 10^{-10} to 10^{-7} M DHT

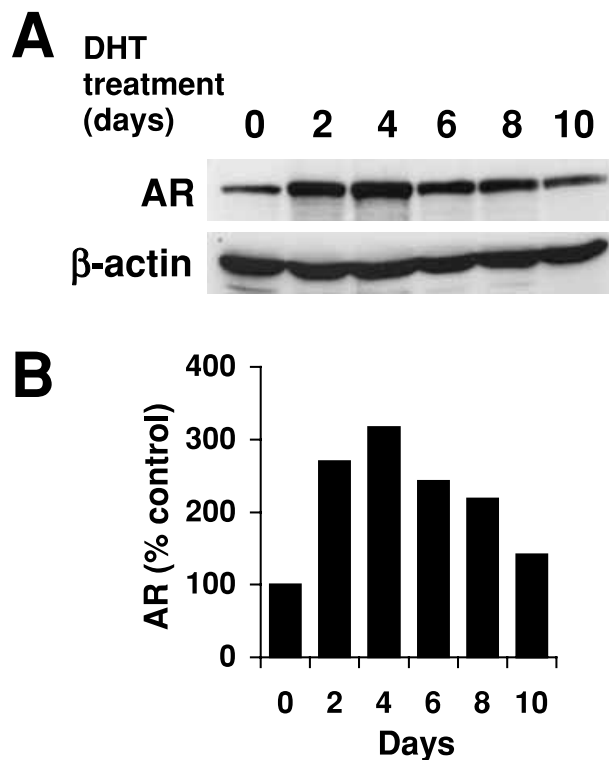


Figure 1 Effects of DHT on AR protein levels. Asynchronously growing MCF-7 cells cultured in RPMI 1640 medium containing 2% FCS were treated with 10^{-8} M DHT for up to 10 days. Whole cell lysates of cultures were separated in 10% polyacrylamide gels. (A) Immunoblotting for the 110 kDa AR (and β -actin to standardise protein loading) indicated an increase in cellular AR levels peaking at 4 days of DHT exposure. (B) Graph of AR protein as a proportion of β -actin for each sample and represented as a percentage of control (0 days) identified elevated AR levels at 2 and 4 days of DHT treatment. Thereafter, AR protein decreased to near baseline (control) levels by 10 days of treatment.

inhibited cell proliferation at all concentrations, with no significant increase in cell numbers after 3 days of treatment ($P > 0.05$). By the end of the treatment period, the mean number of DHT-treated cells was less than 40% of controls at all concentrations of DHT (Fig. 2A). Similarly, treatment of other AR-expressing human breast cancer cell lines (T47-D, ZR-75-1, MDA-MB-453) with 10^{-10} to 10^{-7} M DHT under comparable culture conditions also inhibited proliferation (results not shown). Growth inhibition at 3 days correlated well with the onset of morphological changes (Fig. 2B), with DHT-treated cells

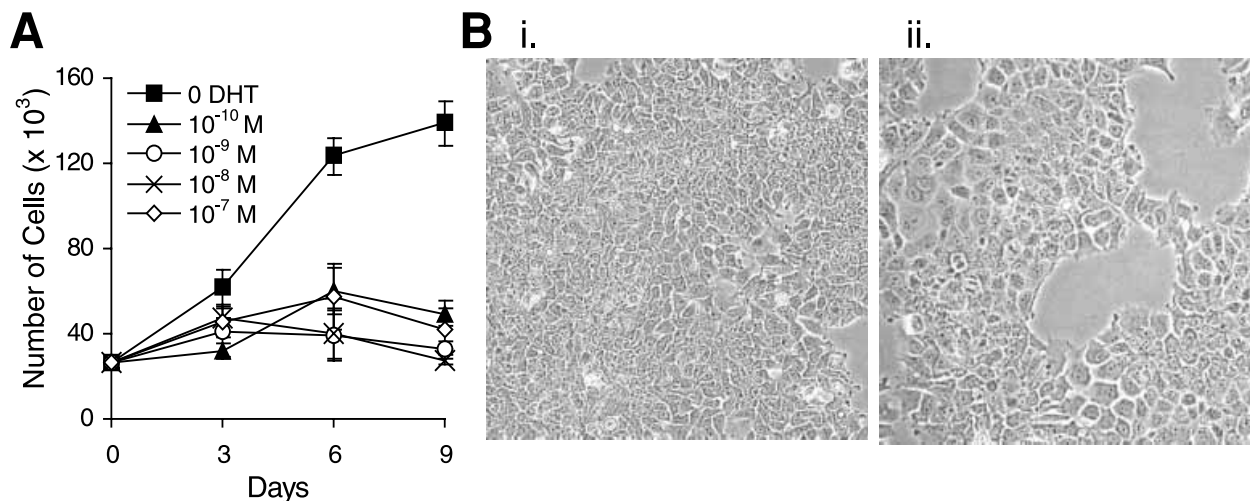


Figure 2 DHT inhibition of MCF-7 cell proliferation. (A) MCF-7 cells were cultured in RPMI 1640 medium containing 2% FCS in the absence or presence of 10⁻¹⁰ to 10⁻⁷ M DHT and cell number in triplicate wells was determined at 3, 6 and 9 days. Treatment of cells with all concentrations of DHT was associated with inhibition of cell proliferation by 3 days of culture. (B) Cellular morphology of (i) untreated MCF-7 cells and (ii) MCF-7 cells treated for 3 days with 10⁻⁸ M DHT. DHT treatment altered the light microscopic appearance of MCF-7 cells, which appeared progressively larger and flattened over the culture surface ($\times 100$).

appearing larger and flattened over the culture surface in comparison with untreated cells.

Since DHT inhibited the growth of MCF-7 cells by 3 days of treatment, flow cytometric analysis was used to determine the cell cycle distribution of DHT-treated cells. DHT treatment for 4 and 7 days induced small (4%–10%), but reproducible, increases in the proportions of G1 phase cells, with corresponding decreases in the proportions of S/G2 phase cells (Fig. 3A). By 7 days of culture in the presence of DHT, the proportion of G1 phase cells was significantly increased compared with controls ($P=0.044$). As only modest increases in G1 phase cells were observed, indicating that DHT does not induce a classical G1 arrest, the effects of DHT on G1 to S phase progression were investigated.

MCF-7 cultures that had been pretreated with 10⁻⁸ M DHT (or vehicle) for 3 days were growth arrested in G1 phase by culturing in serum-free medium for 24 h (+/- DHT). As shown in Fig. 3B, serum starvation of cells for 24 h in the presence or absence of DHT resulted in a similar accumulation of cells in the G1 phase of the cell cycle (89 and 86% respectively). Replacement of culture media with RPMI 1640 medium containing 2% FCS reinitiated cell cycle progression; however, 24 h later 67% of DHT pretreated cells remained in G1

as compared with 45% of control cells. Similarly, 30% of DHT-treated cells were in S phase at this time-point, in contrast to 48% of control cells. These data suggest that DHT acts, at least in part, to impede the progression of cells from G1 into S phase.

Inhibition of G1 Cdk activity and reduced phosphorylation of pRb following androgen treatment

Since growth inhibition by DHT involves impeded progression of cells through the G1 to S phase transition, the effects of DHT treatment on regulators of the restriction point were investigated. Immunoblotting for Rb revealed both phosphorylation forms of the protein, with DHT treatment of asynchronously growing MCF-7 cells associated with decreases in the total amount of Rb protein during 8 days (Fig. 4). Specifically, DHT treatment induced a loss of the hyperphosphorylated form of Rb (ppRb), initially at 2 days, but without any consequent increase in pRb protein. While exponentially growing, control cells expressed equivalent amounts of ppRb and pRb, the latter, inhibitory form of the protein dominated between 2 and 8 days of DHT treatment, comprising 65% of total Rb by 8 days.

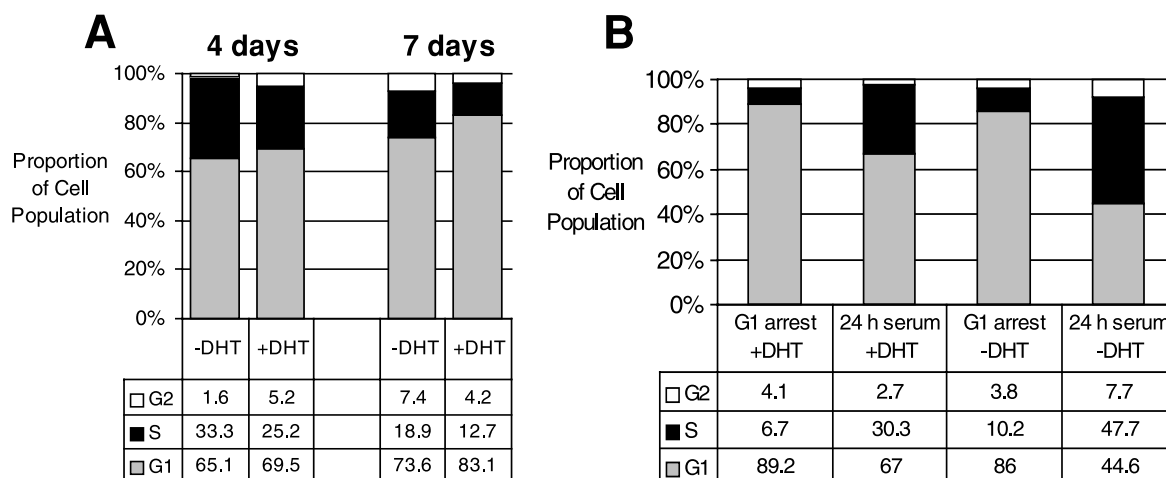


Figure 3 DHT regulation of cell cycle distribution and G1-S phase progression. (A) Cell cycle distribution of MCF-7 cultures treated with DHT. Asynchronously growing cells were cultured in RPMI 1640 medium containing 2% FCS and treated for 4 and 7 days with 10^{-8} M DHT (+) or ethanol vehicle (-). Propidium iodide staining and flow cytometric analysis of cells identified small, but reproducible, increases in the proportion of G1 phase cells in DHT-treated cultures. (B) DHT targets the G1/S phase transition in MCF-7 cells. Cells were arrested in G1 phase ('G1 arrest') by serum deprivation for 24 h in the presence or absence of 10^{-8} M DHT. Cell cycle distribution was assessed by flow cytometry 24 h following addition of medium containing 2% FCS (+/- 10^{-8} M DHT). The increased proportion of DHT-treated cells remaining in G1 phase (67%) as compared with control cultures (44.6%) suggested inhibition of the G1-S phase transition by DHT.

Reduced activity of G1 Cdks is a likely cause of decreased Rb phosphorylation in DHT-treated cells. Both Cdk4 and Cdk2 contribute to the phosphorylation, and hence inhibition, of pRb during the normal passage of cells through G1 and into S phase. The ability of Cdk4 complexes from control and treated cultures to phosphorylate a fragment of Rb was assessed *in vitro*. Cdk4 kinase activity remained close to control levels until 72 h of treatment, at which time activity was reduced to nearly 40% control (Fig. 5A). In contrast, Cdk2 activity, determined by the ability of Cdk2 complexes from control and DHT-treated cultures to phosphorylate histone H1, remained unchanged at 24 h of treatment, before declining to less than 80% at 48 h (Fig. 5B). By 72 h, Cdk2 kinase activity had been reduced to barely detectable levels, representing ~10% control activity. The marked decrease in the activity of both Cdk4 and Cdk2 at 48 and 72 h preceded both the predominance of hypophosphorylated Rb and the DHT-mediated growth arrest of MCF-7 cells. These data suggest that G1 Cdk inhibition is likely to play an important role in the growth-inhibitory properties of DHT in MCF-7 cells.

DHT-mediated growth inhibition is associated with decreased levels of G1 Cdks and D-type cyclins and increased p27^{Kip1} protein levels

Mitogens, such as growth factors and steroid hormones, influence cell proliferation by regulating the expression of cell cycle components including Cdks and cyclins. To investigate possible causes of Cdk4 and Cdk2 inhibition and, therefore, the predominance of inhibitory pRb, the effects of DHT on the protein levels of Cdks and cyclins involved in G1 and S phases were examined.

Cdk4, in association with its binding partners, the D-type cyclins, is involved in the progression of cells through early and mid-G1 phase. Immunoblotting of whole cell extracts over 8 days of DHT treatment indicated that cellular Cdk4 levels were reduced to 80% of controls by 1 day of treatment and to 50% by 8 days (Fig. 6). Similarly, total levels of cyclin D1 were decreased to around 80% of controls following 1 day of treatment and to less than 10% by 8 days. Cyclin D3 demonstrated a less dramatic decline, with subtle decreases evident after 2 days of treatment, with levels falling first to 88% of controls at 2 days and reaching 40% of controls by

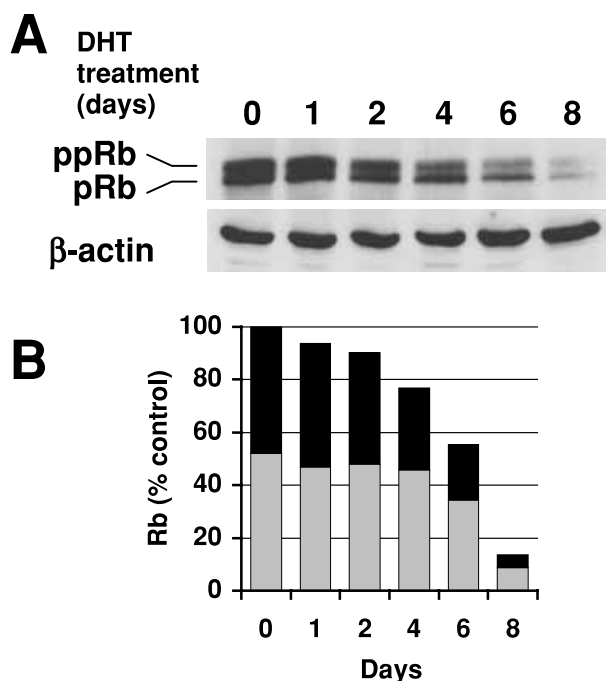


Figure 4 DHT reduces hyperphosphorylated and total Rb protein levels in DHT-treated MCF-7 cells. Asynchronously growing MCF-7 cells were cultured in the presence of 10^{-8} M DHT for up to 8 days. Whole cell extracts were separated in 7.5% polyacrylamide gels prior to immunoblotting for Rb. (A) Immunoblotting for Rb revealed the ppRb (upper) and pRb (lower) forms of the protein. Immunoblotting for β -actin was used to standardise protein loading. (B) Graph of relative proportions of ppRb (solid) and pRb (shaded) represented as a percentage of total Rb protein in control cultures. DHT treatment caused progressive decreases in ppRb protein levels during the first 4 days of treatment followed by a rapid decline in total Rb protein levels.

8 days. These results suggested that reduced expression of key components of Cdk4 complexes may also contribute to the decrease in Cdk4 kinase activity evident at 3 days of DHT treatment.

The progression of cells from G1 into S phase is also dependent on adequate Cdk2/cyclinE complex activity, which is in turn reliant on Cdk2 and cyclin E protein levels. Consistent with the observed reduction in Cdk2 activity, Cdk2 protein levels had decreased to almost 50% by 2 days of treatment, preceding the decrease in kinase activity at 3 days (Fig. 6). In contrast to the D-type cyclins investigated, cyclin E protein levels were unchanged by DHT treatment, with levels remaining close to controls over 8 days. Since the progression

of cells into S phase is dependent on sufficient Cdk2 protein levels, the rapid fall in the level of this protein in response to DHT treatment is likely to contribute to decreased Cdk2 kinase activity and, consequently, an impeded G1 to S phase transition.

In addition to changes in cyclin and Cdk protein expression, the association of Cdks with Cdkis such as p21^{Cip1/Waf1} and p27^{Kip1} regulates their activity. Since the gene encoding the p21^{Cip1/Waf1} protein contains a putative androgen response element within its promoter (Lu *et al.* 1999) and may therefore be androgen responsive in human breast cancer cells, immunoblotting for p21^{Cip1/Waf1} in DHT-treated MCF-7 cells was performed. However, Fig. 7 illustrates that p21^{Cip1/Waf1} protein remained at almost control levels throughout the 8 days of treatment, suggesting that growth inhibition by DHT is not mediated by changes in p21^{Cip1/Waf1} protein levels.

Changes in p27^{Kip1} expression and its association with Cdk2/cyclin E complexes play an important role in the regulation of normal breast epithelial cell growth and development (Muraoka *et al.* 2001) as well as breast cancer cell proliferation (Menjo *et al.* 1998, Loden *et al.* 1999, Cariou *et al.* 2000, Swarbrick *et al.* 2000, Lapointe & Labrie 2001). Similarly, p27^{Kip1} levels are elevated during growth inhibition of LNCaP prostate cancer cells by high-dose androgen treatment, and are thought to play a pivotal role in this G1 growth arrest (Tsihlias *et al.* 2000). In order to determine if p27^{Kip1} is involved in the inhibition of MCF-7 breast cancer cell proliferation by DHT, immunoblotting for p27^{Kip1} protein was performed. Treatment of cells with 10^{-8} M DHT was associated with an increase in the cellular levels of this protein, initially within 1 day of treatment, with levels peaking at 230% of controls at 4 and 6 days before declining (Fig. 7). Consistent with changes in the expression of Cdks and cyclins, the initial increases in p27^{Kip1} protein at 1 and 2 days preceded the inhibition of Cdk2 and Cdk4 kinase activity evident at 2 and 3 days, suggesting a possible causative role in this inhibition.

Changes in p21^{Cip1/Waf1} association with Cdk4- and Cdk2-associated complexes during DHT treatment

Cdk4/cyclin D complexes are key regulators of G1 progression and are frequently targeted for

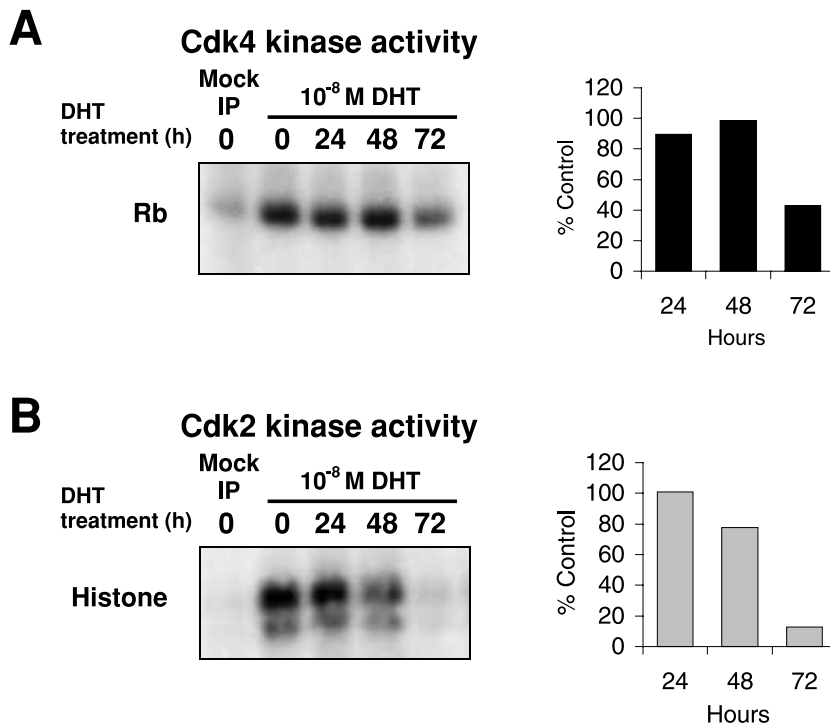
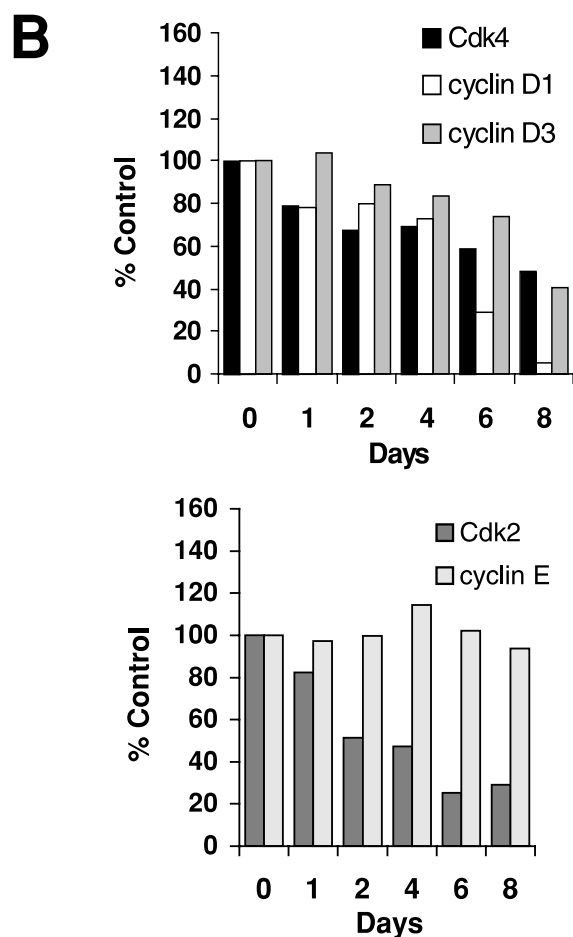
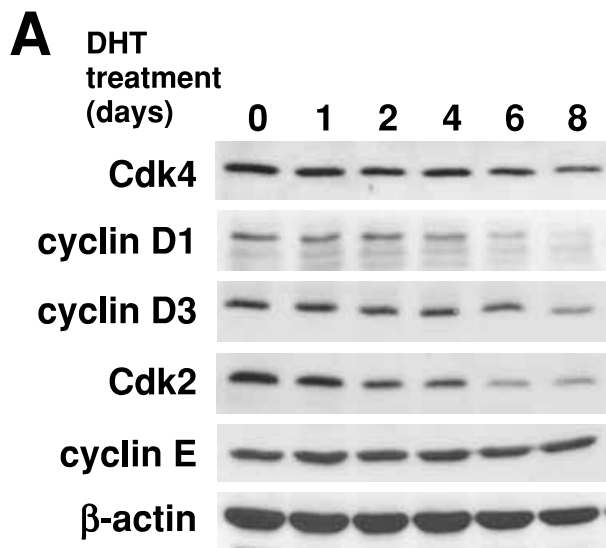


Figure 5 DHT inhibits G1 Cdk kinase activity. MCF-7 cells cultured in RPMI 1640 medium containing 2% FCS were treated for up to 72 h with 10⁻⁸ M DHT for *in vitro* analysis of kinase activities of G1 phase Cdks. (A) Cdk4 kinase activity. Immunoprecipitated Cdk4 complexes were assayed for kinase activity using Rb (amino acids 773–928) as the substrate (left). Following subtraction of background ('mock IP'), comparative band densities indicated little change in Cdk4 kinase activities at 24 and 48 h with Cdk4 kinase activity reduced to approximately 40% of control at 72 h (right). (B) Cdk2 kinase activity. Immunoprecipitated Cdk2 complexes were assayed for phosphorylation of histone H1 (left). DHT treatment was associated with decreases in Cdk2 kinase activity to 80 and 10% of controls at 48 and 72 h of treatment respectively (right).

inhibition by growth-suppressive mitogens. In order for G1 phase to proceed, Cdk4 must form active kinase complexes with its binding partners, the D-type cyclins, and avoid association with saturating levels of inhibitors such as p21^{Cip1/Waf1}. In order to determine if DHT reduces the association of D-type cyclins with Cdk4, changes in the composition of Cdk4 complexes were assessed by immunoprecipitation. Immunoblots for key components of Cdk4 complexes during 48 h of DHT treatment are shown in Fig. 8A. Although DHT treatment reduced total cyclin D1 protein levels after 1 day (Fig. 5A), there were no detectable changes in the association of this cyclin with Cdk4 during 48 h of treatment. In contrast, however, Cdk4 immunoprecipitates contained relatively

constant amounts of cyclin D3 until 24 h of treatment, after which Cdk4-bound cyclin D3 fell to 70% of controls at 48 h (standardised against immunoprecipitated Cdk4).

In addition to inhibiting Cdk activity at saturating concentrations, p21^{Cip1/Waf1} has recently been shown to perform a vital role in the assembly of Cdk4 protein complexes and, in particular, the association of the D-type cyclins with Cdk4 (LaBaer *et al.* 1997, Cheng *et al.* 1999, Russell *et al.* 1999). Consistent with a role in Cdk4/cyclin D(1/3) assembly, p21^{Cip1/Waf1} was present in Cdk4 protein complexes in asynchronously growing MCF-7 cells (Fig. 8A). Similarly, immunoprecipitation of Cdk4 from cell lysates removed a considerable proportion (~90%) of



total p21^{Cip1/Waf1}, suggesting that the protein is present in Cdk4 complexes in control cells where it is likely to have a non-inhibitory function (Fig. 8B).

Investigation of Cdk4-associated complexes during 48 h of DHT treatment showed a decrease in association with p21^{Cip1/Waf1} beginning at 24 h. When standardised against immunoprecipitated Cdk4, Cdk4-associated p21^{Cip1/Waf1} decreased further to 75% of controls at 48 h; a result analogous to the fall in Cdk4-associated cyclin D3 levels described above (Fig. 8A). Analysis of cell lysates following immunoprecipitation of Cdk4 complexes demonstrated that the Cdk4 complexes contained a high proportion of total cellular p21^{Cip1/Waf1} until 24 h of treatment, at which time there was a considerable increase in non-Cdk4-associated p21^{Cip1/Waf1} (Fig. 8B). Similarly, p27^{Kip1}, which has recently been shown to be essential for Cdk4/cyclin D complex formation and kinase activity (Cheng *et al.* 1999), remained largely in Cdk4 complexes until 24 h of treatment, with Cdk4 likely to sequester this potential Cdk2 inhibitor in proliferating cells. During 48 h of DHT treatment, depletion of Cdk4 removed decreasing amounts of the inhibitor, suggesting progressive dissociation of p27^{Kip1} from Cdk4 complexes.

Since DHT treatment was associated with a decrease in cellular Cdk2 levels but not cyclin E levels, Cdk2 immunoprecipitates were assessed for changes in association with cyclin E and p21^{Cip1/Waf1} (Fig. 9A). Densitometry analysis of immunoblots revealed that, during 48 h of treatment, Cdk2-associated cyclin E remained at almost control levels. In contrast, increases in the association of Cdk2 with the inhibitor, p21^{Cip1/Waf1}, were detected during DHT treatment,

Figure 6 Effects of DHT on G1 phase Cdk and cyclin protein levels. Asynchronously growing MCF-7 cells were cultured in RPMI-1640 medium containing 2% FCS and treated with 10⁻⁸ M DHT for up to 8 days. Whole cell extracts were separated in 12% polyacrylamide gels for immunoblot analysis. (A) Immunoblots for Cdk4 and its associated cyclins D1 and D3, and Cdk2 and cyclin E. β-actin immunoblots were used to standardise protein loading in each lane. (B) Relative protein levels determined by densitometry analysis of immunoblots are expressed as percentage of controls (0 days). DHT treatment was associated with progressive reductions in the levels of Cdk4, Cdk2, cyclin D1 and cyclin D3. Cyclin E levels were not altered during 8 days of DHT treatment.

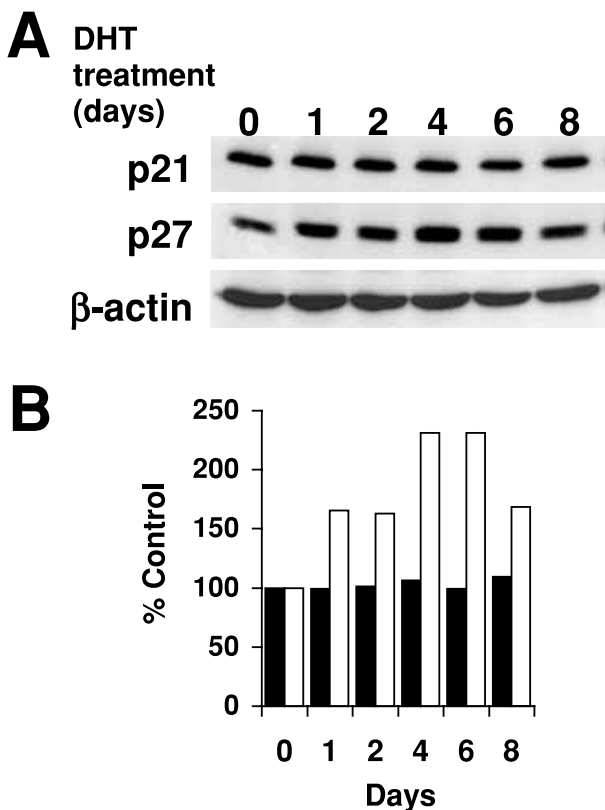


Figure 7 DHT treatment increases p27^{Kip1} but not p21^{Cip1/Waf1} protein levels. p21^{Cip1/Waf1} and p27^{Kip1} protein levels were analysed by immunoblotting of asynchronously growing MCF-7 cells cultured for up to 8 days in the presence of 10⁻⁸ M DHT. (A) Representative immunoblots for p21^{Cip1/Waf1} (p21), p27^{Kip1} (p27) and β-actin. (B) Relative protein levels of p21^{Cip1/Waf1} (solid bars) and p27^{Kip1} (open bars) as determined by densitometry with standardisation of band densities against the β-actin blot. Results are expressed as a percentage of controls and indicate increased p27^{Kip1} protein levels at 1–8 days of DHT treatment. In contrast, no change in p21^{Cip1/Waf1} levels was detected during this time-period.

with Cdk2-associated p21^{Cip1/Waf1} increasing to almost 160% of control levels by 24 h and remaining at 140% by 48 h. Similarly, immunoprecipitation of Cdk2 complexes removed 45% of total p21^{Cip1/Waf1} from untreated lysates with 48 h of DHT treatment associated with co-depletion of progressively more p21^{Cip1/Waf1} protein (Fig. 9B). In contrast, immunoprecipitation of Cdk2 from the lysates of DHT-treated MCF-7 cells was not associated with increases in the proportion of co-depleted p27^{Kip1}. Interestingly, immunocytochemical staining for p27^{Kip1} protein in both the

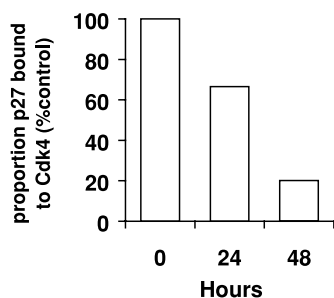
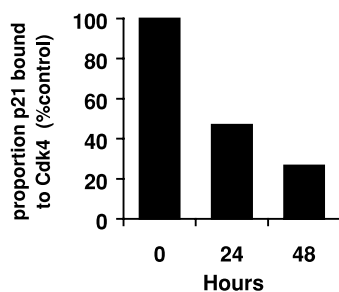
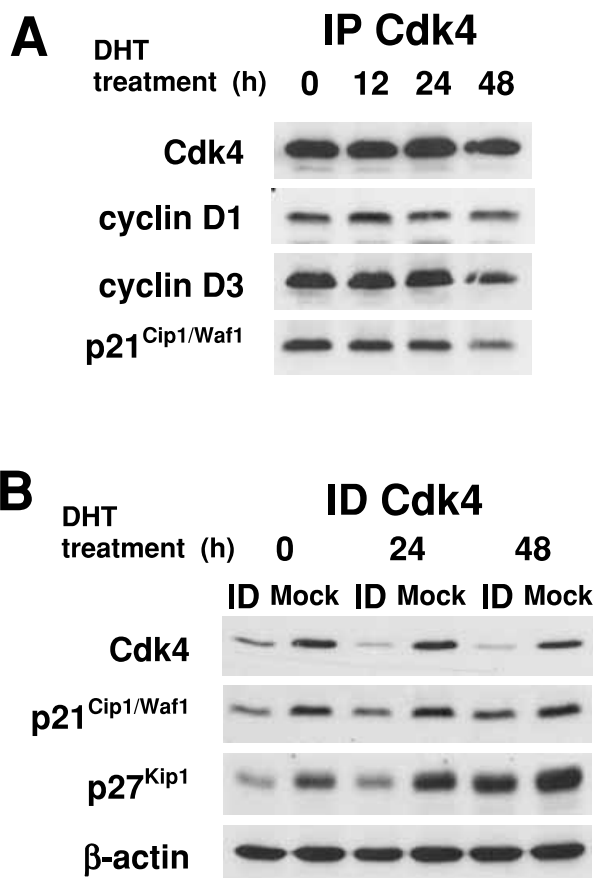
cytoplasm and nucleus is increased during DHT treatment of MCF-7 cells without noticeable increases in the proportion of nuclear staining (data not shown), suggesting that p27^{Kip1} may be sequestered away from Cdk2 complexes in the cytoplasm.

The enhanced association of Cdk2 with p21^{Cip1/Waf1} and the corresponding loss of p21^{Cip1/Waf1} from Cdk4 complexes preceded the reduction in Cdk2 kinase activity seen at 48 and 72 h of DHT treatment. Together, these results suggest that the growth-inhibitory properties of DHT in MCF-7 breast cancer cells are mediated, at least in part, by changes in p21^{Cip1/Waf1} and cyclin D3 association with Cdk4 and Cdk2 and the subsequent inhibition of these kinases.

Discussion

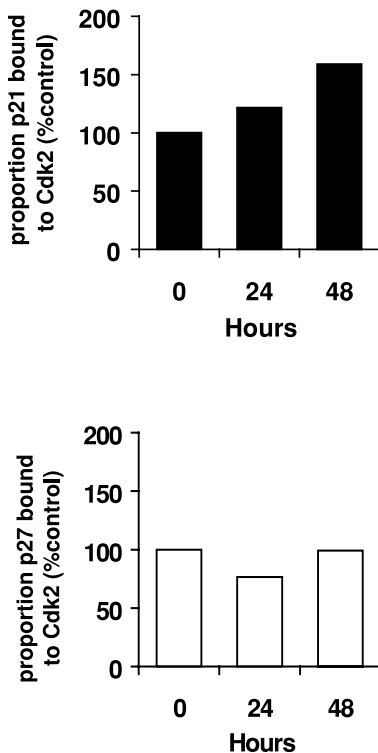
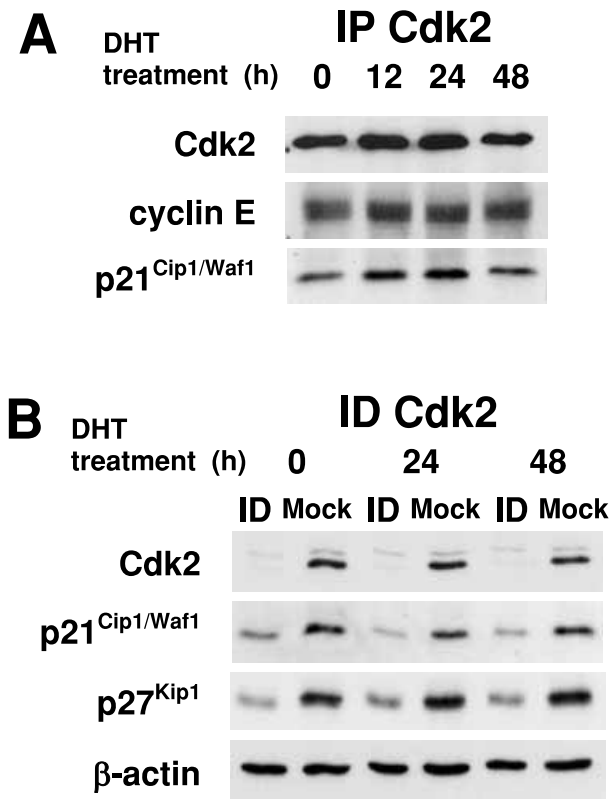
Expression of the AR by breast tumours correlates with better prognosis; however, few studies have addressed the molecular mechanisms of androgen action in breast cancer cells. In the current study, we have shown that androgen treatment of MCF-7 breast cancer cells is associated with increased AR protein levels and inhibition of cell proliferation within 3 days of treatment. The lack of increase in cell numbers during the treatment period was not associated with a classical G1 arrest (~90% of cells in G1) but was accompanied by increases of <10% in the proportion of G1 phase cells with corresponding decreases in the percentage of cells in S phase. These findings are in contrast to a previous analysis of growth inhibition of prostate cancer cells induced by high-dose androgen treatment (Tsihlias *et al.* 2000); however, the results are consistent with a number of reports of androgen activity in breast cancer cells (de Launoit *et al.* 1991, Yeap *et al.* 1999, Lapointe & Labrie 2001). As several different breast cancer cell lines have been used in these studies and androgen inhibition of both oestrogen- and serum-induced proliferation has been documented in the present and in previous reports, the findings provide evidence of a mechanism of androgen activity that is characteristic of breast cancer cells and, at least in part, distinct from its mechanism of action in prostate-derived or prostate cancer cells.

Androgens have been shown to induce anti-oestrogenic effects in breast cancer cells, principally



by decreasing ER expression (Poulin *et al.* 1989, Zhou *et al.* 2000). However, growth suppression by androgens is thought to be additive to that of anti-oestrogens (Dauvois *et al.* 1991), suggesting that the steroids also induce changes independent from oestrogen responsiveness. Until recently, the effects of androgens such as DHT on the cell cycle have remained unclear. Using ZR-75-1 human breast cancer cells, de Launoit *et al.* (1991) noted that DHT caused a global slowing of the cell cycle without changes in cell cycle distribution. DHT treatment of the AR-expressing breast cancer cell line, MDA-MB-453, is associated with a decreased proportion of cells in S phase (Yeap *et al.* 1999) and similar alterations in the proportions of G1 and S phase cells to the present study have been documented in association with inhibition of oestrogen-stimulated growth of CAMA-1 breast cancer cells by DHT (Lapointe & Labrie 2001). At a superphysiological concentration of 10^{-7} M, DHT has been shown to impede MCF-7 breast cancer cell proliferation in the presence or absence of 17β -oestradiol and increase the number of cells in G0/G1 via a mechanism involving the AR (Ando *et al.* 2002). This study complemented earlier

Figure 8 Effects of DHT on Cdk4-associated complexes. Asynchronously growing MCF-7 cells were cultured in RPMI 1640 medium containing 2% FCS and treated for up to 48 h with 10^{-8} M DHT. (A) Immunoblots for Cdk4-associated proteins. Cell lysates were prepared from control and DHT-treated cultures and Cdk4 complexes were precipitated from lysates using protein G-sepharose following overnight incubation with anti-Cdk4. Captured proteins were separated in 14% polyacrylamide gels and immunoblotted for Cdk4, cyclin D1, cyclin D3 and p21^{Cip1/Waf1}. The results show decreasing association of Cdk4 with cyclin D3 and p21^{Cip1/Waf1} during 48 h of DHT treatment. (B) Immunodepletion (ID) of Cdk4 complexes. Cdk4 was immunoprecipitated from lysates as described above, and the Cdk4-depleted protein supernatants were separated in 14% polyacrylamide gels and immunoblotted for Cdk4, p21^{Cip1/Waf1} and p27^{Kip1}. Relative amounts of non-Cdk4-associated p21^{Cip1/Waf1} and p27^{Kip1} were calculated by densitometry analysis of immunoblots with 'mock ID' samples representing total protein present in cell extracts. Results were corrected for immunodepletion using the Cdk4 blot, standardised against β -actin and expressed as a percentage of total p21^{Cip1/Waf1} (p21; solid bars; upper graph) or p27^{Kip1} (p27; open bars; lower graph) bound to Cdk4. Levels of both Cdk4-associated p21^{Cip1/Waf1} and p27^{Kip1} decreased during DHT treatment of MCF-7 cells.



research conducted by Szelei *et al.* (1997) using MCF-7 cells transfected with the AR, which express five times wild-type AR levels. These highly androgen-sensitive cells arrested in G0/G1 phase when exposed to 3×10^{-9} M of the synthetic androgen, R1881. Thus, although physiological levels of DHT do not appear to cause a G1 arrest of asynchronously growing cells, it is clear that DHT impedes progression of breast cancer cells from G1 into S phase.

The normal transition of cells through the G1/S phase transition is regulated by the activity of pocket proteins such as Rb. In the absence of G1 Cdk kinase activity, Rb remains hypophosphorylated and binds E2F transcription factors, thus preventing the expression of genes required for S phase entry. Both Cdk4 and Cdk2, in association with their cyclin partners, phosphorylate Rb at specific residues during the passage of cells through G1 phase (Connell-Crowley *et al.* 1997, Lundberg & Weinberg 1998). While studies have attempted to identify the specific Cdk/cyclin complex responsible for the inactivation of Rb, a theory by which the sequential and obligatory phosphorylation of Rb by Cdk4/cyclin D and Cdk2/cyclin E respectively has emerged as the most likely scenario

Figure 9 Alterations in Cdk2 complexes during DHT treatment. Asynchronously growing MCF-7 cells were cultured in RPMI 1640 medium containing 2% FCS and treated for up to 48 h with 10^{-8} M DHT. (A) Immunoblotting for Cdk2-associated proteins. Cell lysates were prepared from control and DHT-treated cultures and Cdk2 complexes were precipitated from lysates using protein G-sepharose following overnight incubation with anti-Cdk2 (68476E). Captured proteins were separated in 14% polyacrylamide gels. Immunoblotting for Cdk2, cyclin E and p21^{Cip1/Waf1} indicated increasing association of Cdk2 with p21^{Cip1/Waf1} until 24 h but no changes in Cdk2-bound cyclin E. (B) Immunoprecipitation of Cdk2 was performed as described above and Cdk2-depleted lysates were immunoblotted for Cdk2, p21^{Cip1/Waf1} and p27^{Kip1}. Relative amounts of non-Cdk2-associated p21^{Cip1/Waf1} and p27^{Kip1} were calculated by densitometry analysis of immunoblots with 'mock ID' samples representing total protein present in cell extracts. Results were corrected for immunodepletion using the Cdk2 blot, standardised against β-actin and expressed as a percentage of total p21^{Cip1/Waf1} (p21; solid bars; upper graph) or p27^{Kip1} (p27; open bars; lower graph) bound to Cdk2. Increases in the association of p21^{Cip1/Waf1} with Cdk2 were confirmed while Cdk2-associated p27^{Kip1} protein remained close to control levels throughout 48 h of DHT treatment.

(Lundberg & Weinberg 1998, Harbour *et al.* 1999). Consistent with observed decreases in the proportion of hyperphosphorylated Rb in the present study, DHT treatment attenuated both Cdk4- and Cdk2-associated kinase activities within 3 days.

It is not surprising that the relative levels of G1 Cdk and cyclins are rate limiting for G1-S phase progression since the expression of both of these components is pertinent for Rb phosphorylation (Resnitzky *et al.* 1994, Resnitzky & Reed 1995, Herzinger & Reed 1998). By immunoblotting for key Cdk and cyclins that normally participate in Rb phosphorylation, we have demonstrated that DHT treatment is associated with decreases in the steady-state levels of Cdk4 and Cdk2 proteins, as well as those of the D-type cyclins. The expression of D-type cyclins is often altered in response to mitogens, and cyclin D1 is upregulated during exposure of human breast cancer cells to oestrogens (Altucci *et al.* 1996, Planas-Silva & Weinberg 1997). Despite decreases in the levels of cyclin D1 in DHT-treated MCF-7 cells, analysis of immunoprecipitated Cdk4 complexes failed to reveal changes in Cdk4 association with this cyclin. Although this is unexpected, cyclin D1 is overexpressed by MCF-7 cells (Russell *et al.* 1999) and may not be limiting for Cdk4/cyclin D1 complex formation, particularly as DHT treatment also reduces Cdk4 protein levels. In contrast, decreases in cyclin D3 protein levels coincided with its reduced association with Cdk4, suggesting that DHT may impede the progression of MCF-7 cells through the G1/S phase transition by suppressing Cdk4/cyclin D3 complex formation.

In addition to Cdk/cyclin complex abundance, G1 phase progression is regulated by the interaction of Cdk with Cdkis including p21^{Cip1/Waf1} and p27^{Kip1}. In many tissue culture systems, the levels of these inhibitors are thought to provide thresholds for the activity of Cdk, thereby influencing the duration of the cell cycle. Similarly, in the present study, the treatment of MCF-7 cells with DHT was accompanied by a more than twofold increase in p27^{Kip1} protein levels by 4 days, which was likely to increase the threshold for Cdk activity and delay the onset of S phase. These observations are in agreement with the work of Lapointe & Labrie (2001) who noted similar changes in p27^{Kip1} levels during the treatment of CAMA-1 breast cancer cells with DHT. p27^{Kip1} is a more potent inhibitor of Cdk2/cyclin A than

Cdk4/cyclin D complexes (Blain *et al.* 1997) and is likely to interact with Cdk2/cyclin E in DHT-treated cells in a similar manner. Interestingly, however, active Cdk2/cyclin E complexes are also involved in regulating p27^{Kip1} levels by directly phosphorylating the protein on Thr187 to facilitate its elimination from the cell (Sheaff *et al.* 1997). It is conceivable, therefore, that the elevation in p27^{Kip1} levels seen during DHT treatment may result from deregulated p27^{Kip1} proteosomal degradation as a consequence of Cdk2/cyclin E inhibition and that p27^{Kip1} does not participate in the inhibition of Cdk2 or Cdk4 complexes during the initial 48 h of DHT treatment.

Another member of the Cip/Kip family, p21, contains an androgen response element within its promoter and its expression is upregulated during androgen treatment of prostate cancer cells (Lu *et al.* 1999, 2000). Similarly, p21^{Cip1/Waf1} expression is increased in MCF-7 cells when treated with anti-oestrogens and the protein plays a key role in this growth arrest (Cariou *et al.* 2000, Carroll *et al.* 2000, Skildum *et al.* 2001). Given that p21^{Cip1/Waf1} is androgen regulated in other systems (Lu *et al.* 1999, 2000), we hypothesised that the protein may also be involved in the androgen-mediated inhibition of breast cancer cell proliferation. Nevertheless, in contrast to p27^{Kip1}, p21^{Cip1/Waf1} levels remained relatively constant during 8 days of DHT treatment, although we have found that DHT treatment of MCF-7 cells cultured in steroid-depleted medium causes upregulation of p21^{Cip1/Waf1} levels (K Liyanage & J M Bentel, unpublished observations). As p21^{Cip1/Waf1} protein levels are high in the MCF-7 cells used in this study and reduced in cultures grown in steroid-depleted medium containing charcoal-treated FCS, these findings suggest that asynchronous growth of MCF-7 occurs in the presence of high p21^{Cip1/Waf1} levels that are induced by serum factors and that DHT treatment is not able to further increase levels of p21^{Cip1/Waf1}.

Analysis of Cdk4 complex composition revealed progressive decreases in the abundance of Cdk4-associated p21^{Cip1/Waf1}. Significantly, this release of p21^{Cip1/Waf1} from Cdk4 coincided with its increased association with Cdk2, suggesting that DHT may initiate the redistribution of the inhibitor between Cdk/cyclin complexes. Since p21^{Cip1/Waf1} is more frequently observed as an inhibitor of Cdk2/cyclin E than Cdk4/cyclin D, it has been

suggested that Cdk4 complexes may act to titrate this potential inhibitor away from Cdk2/cyclin E late in G1 phase. Indeed, p21^{Cip1/Waf1} expression appears necessary for the assembly and stabilisation of D-type cyclins with Cdk4 (LaBaer *et al.* 1997, Cheng *et al.* 1999, Parry *et al.* 1999), and immunodepletion of Cdk4 from control lysates in the present study removed a marked proportion of p21^{Cip1/Waf1}. However, at 24 and 48 h of treatment, the reservoir of non-Cdk4-associated p21^{Cip1/Waf1} increased and is likely to represent the increased association of the inhibitor with Cdk2. Similarly, immunodepletion experiments revealed that levels of Cdk4-associated p27^{Kip1}, which were high in untreated cells, were reduced most markedly at 48 h of DHT treatment, suggesting a more delayed response in comparison with p21^{Cip1/Waf1}.

The mechanism by which DHT liberates p21^{Cip1/Waf1} and p27^{Kip1} from Cdk4/cyclin D may involve the participation of INK4 inhibitors. These proteins can both bind monomeric Cdks to prevent cyclin attachment and inhibit the phosphorylation and hence activation of pre-formed Cdk/cyclin complexes (Jeffrey *et al.* 2000). Several studies have suggested that INK4 inhibitors are also involved in the redistribution of Cip/Kip family members and co-operate with these inhibitors to suppress Cdk activity. The upregulation of p15^{INK4a} in multiple cell types in response to transforming growth factor- β , for instance, is associated with the uncoupling of cyclin D1, p21^{Cip1/Waf1} and p27^{Kip1} from Cdk4 complexes and the redistribution of p27^{Kip1} to Cdk2/cyclin E (Reynisdottir *et al.* 1995, Reynisdottir & Massague 1997, Sandhu *et al.* 1997). Similarly, the binding of p16^{INK4b} to Cdk4/6 in U2-OS osteogenic sarcoma cells initiates the transfer of p21^{Cip1/Waf1} to Cdk2 complexes and blocks the association of cyclin D1, p21^{Cip1/Waf1} and p27^{Kip1} with Cdk4/6 (Mittra *et al.* 1999). Since INK4 proteins induce cyclin displacement from pre-formed Cdk/cyclin complexes and may prevent their reassociation (Sandhu *et al.* 1997), these molecules are thought to antagonise the role of Cip/Kip proteins in Cdk4/cyclin D assembly and stabilisation, thereby providing thresholds for inhibition during normal cell proliferation (Parry *et al.* 1999). As such, the inhibition of MCF-7 cell proliferation by DHT may involve the association of INK4 inhibitors with Cdk4/cyclin D3/p21^{Cip1/Waf1} complexes, which

then facilitate the dissociation of both cyclin D3 and p21^{Cip1/Waf1}. MCF-7 cells do not express functional p16^{INK4b} and previous studies have not documented a role for INK4 inhibitors in androgen-mediated growth inhibition (Tsihlias *et al.* 2000, Lapointe & Labrie 2001). Further experiments are required to investigate the involvement of INK4 proteins in the effects of DHT on the cell cycle in breast cancer or other cell types.

Several reports have implicated p21^{Cip1/Waf1} in the inhibition of breast cancer cell proliferation by various agents (Planas-Silva & Weinberg 1997, Gooch *et al.* 2000, Lai *et al.* 2001). Although p21^{Cip1/Waf1} is overexpressed in the MCF-7 cell line, surplus inhibitor is titrated away from Cdk2 complexes by similarly elevated cyclin D3 levels (Russell *et al.* 1999). As a consequence, overexpression of cyclin D3 and its association with p21^{Cip1/Waf1} contribute to enhanced Cdk2 activity in MCF-7 cells (Russell *et al.* 1999). DHT-induced decreases in cyclin D3 levels, therefore, are likely to liberate high levels of p21^{Cip1/Waf1} otherwise sequestered by these cyclin complexes. Given that the association of Cdk2 with one molecule of p21^{Cip1/Waf1} is sufficient for complete inhibition (Hengst *et al.* 1998), the redistribution of p21^{Cip1/Waf1} from Cdk4/cyclin D to Cdk2/cyclin E complexes during DHT treatment is likely to render a marked proportion of the kinase inactive. Similar redistribution of p21^{Cip1/Waf1} to Cdk2 has been associated with a loss of Cdk2-associated kinase activity. Recruitment of p21^{Cip1/Waf1} to Cdk2/cyclin E following 6 h of treatment of MCF-7 cells with the anti-oestrogen, ICI 182780, for instance, is initiated prior to the association of Cdk2/cyclin E complexes with p27^{Kip1} and coincides with decreased cyclin E-associated kinase activity (Carroll *et al.* 2000). Likewise, the transient association of p21^{Cip1/Waf1} with Cdk2 precedes increases in Cdk2-associated p27^{Kip1} during interferon- α treatment of Daudi cells, correlating with loss of Cdk2-associated kinase activity (Sangfelt *et al.* 1999). In the present study, Cdk2 kinase activity is reduced at 48 h, following the recruitment of p21^{Cip1/Waf1} to the kinase, suggesting the involvement of p21^{Cip1/Waf1} in Cdk2 inhibition.

The role of p21^{Cip1/Waf1} in androgen-mediated growth inhibition of breast cancer cells has not been reported previously. The findings in this study have indicated that redistribution of p21^{Cip1/Waf1}

from Cdk4 complexes and its increased association with Cdk2 complexes contribute to androgen-mediated inhibition of proliferation of MCF-7 cells. The widespread expression of the AR in human primary and metastatic breast tumours and accumulating evidence of the potent growth-inhibitory activity of androgens in breast cancer cells (de Launoit *et al.* 1991, Birrell *et al.* 1995, Yeap *et al.* 1999, Lapointe & Labrie 2001) support the targeting of the AR pathway in treatments of early stage and advanced disease. In particular, the increased expression of D-type cyclins in MCF-7 cells used in this study did not negate the growth-inhibitory effects of DHT, indicating the potential efficacy of androgens in the treatment of breast tumours that frequently and aberrantly overexpress these key regulators of the G1-S transition.

Acknowledgements

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