A Pure Estrogen Antagonist Inhibits Cyclin E-Cdk2 Activity in MCF-7 Breast Cancer Cells and Induces Accumulation of p130-E2F4 Complexes Characteristic of Quiescence*

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Estrogen antagonists inhibit cell cycle progression in estrogen-responsive cells, but the molecular mechanisms are not fully defined. Antiestrogen-mediated G1/G0 arrest is associated with decreased cyclin D1 gene expression, inactivation of cyclin D1-cyclin dependent kinase (Cdk) 4 complexes, and decreased phosphorylation of the retinoblastoma protein (pRb). We now show that treatment of MCF-7 breast cancer cells with the pure estrogen antagonist ICI 182780 results in inhibition of cyclin E-Cdk2 activity prior to a decrease in the G1 to S phase transition. This decrease was dependent on p21WAF1/Cip1 since treatment with antisense oligonucleotides to p21 attenuated the effect. Recruitment of p21 to cyclin E-Cdk2 complexes was in turn dependent on decreased cyclin D1 expression since it was apparent following treatment with antisense cyclin D1 oligonucleotides. To define where within the G0 to S phase continuum antiestrogen-treated cells arrested, we assessed the relative abundance and phosphorylation state of pocket protein-E2F complexes. While both pRb and p107 levels were significantly decreased, p130 was increased 4-fold and was accompanied by the formation of p130/E2F4 complexes and the accumulation of hyperphosphorylated E2F4, putative markers of cellular quiescence. Thus, ICI 182780 inhibits both cyclin D1-Cdk4 and cyclin E-Cdk2 activity, resulting in the arrest of MCF-7 cells in a state with characteristics of quiescence (G0), as opposed to G1 arrest.

Despite the fact that antiestrogens are widely employed in the treatment of hormone-responsive breast cancers, the molecular mechanisms by which this diverse class of compounds inhibit cellular proliferation are not fully defined. Tamoxifen, the current endocrine therapy of choice in early and advanced breast cancer, leads to highly significant decreases in the rates of both disease recurrence and death (1, 2), but tamoxifen therapy is limited by the inevitable development of cellular resistance (3, 4). Since synthetic non-steroidal antiestrogens like tamoxifen possess both estrogen agonist and antagonist activity (5) more selective steroidal estrogen antagonists have been developed (6). One such compound, the pure steroidal antiestrogen ICI 182780, is currently being used in trials in the treatment of tamoxifen-resistant breast cancers (7, 8).

Both tamoxifen and ICI 182780 bind to the estrogen receptor (ER),† a member of the nuclear receptor superfamily of transcription factors (9, 10). Like other members of this superfamily, the ER activates transcription via two transactivation function domains, located in the N-terminal (AF-1) and C-terminal ligand-binding region (AF-2). Steroidal and nonsteroidal antiestrogens induce different conformational changes in the ER resulting in the recruitment of distinct subsets of transcriptional co-activators and co-repressors (11, 12). It is the differential association of these co-activators and co-repressors that is thought to contribute to the inhibition of only AF-2 by tamoxifen and inhibition of both AF-1 and AF-2 by ICI 182780 (13). Presumably as a consequence of the inhibition of ER-mediated gene transcription, antiestrogen treatment of proliferating ER-positive breast cancer cells arrests cell cycle progression with accumulation of cells in the G0/G1 phase of the cell cycle (14–17).

Cellular proliferation is mediated by the action of a family of serine/threonine kinases, the cyclin-dependent kinases (CDKs), which in conjunction with their regulatory partners, cyclins, phosphorylate pRb and other members of the pocket protein family, p107 and p130 (18, 19). The phosphorylation of pRb during G1 phase is mediated via two temporally distinct stages, in which initial phosphorylation by cyclin D1-Cdk4/6 is followed by cyclin E-Cdk2 phosphorylation to complete inactivation of pRb (20, 21). A major level of cyclin-CDK regulation is from two families of specific inhibitors. The INK4 inhibitors, p16INK4a, p15INK4b, p18INK4c, and p19INK4d, have as primary targets Cdk4 and Cdk6 (22). Members of the second family, which include p21WAF1/Cip1 and p27Kip1,† have a dual function: inhibition of CDKs including cyclin E-Cdk2 as well as facilitation of the assembly of cyclin D-Cdk4/6 complexes (22).

The association of the pocket proteins with members of the E2F family of transcription regulators is responsible for a major part of their growth inhibitory actions. Phosphorylation and hence, inactivation, of the pocket proteins results in dissociation of E2F, allowing subsequent transcription of genes required for progression through late G1 and S phase (23–25). While the availability of “free” E2F is an important determinant of transcriptional activity, it is also apparent that pocket protein-E2F complexes can actively repress transcription of...
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target genes, in addition to simply blocking the action of E2F (23–25). Specific complexes between different E2F family members and pocket proteins are characteristic of particular stages of the cell cycle. During G1 phase, pRb preferentially complexes with E2Fs 1–3, although pRb-E2F4 complexes are present in some cells. p107 is not abundant or active until late G1, where it negatively regulates E2F4 and E2F5 at the G1 to S phase boundary (23–25). p130 is the predominant pocket protein in quiescent (G0) and differentiated cells and interacts with E2F4 in these cells (23–25). The abundance of these complexes is regulated not only by the phosphorylation of the pocket proteins, but also by the levels of both component proteins. p107 and p130 are often reciprocally regulated, contributing to the shift of E2F4 from p130 to p107 during exit from quiescence (26). E2Fs 1–3 are induced during passage through G1, and thus are available to bind pRb and p107 (26).

Our previous work on the mechanisms of antiestrogen-induced cell cycle arrest in hormone-responsive breast cancer cells focused on the role of cyclin D1-Cdk4 and showed a 40% decrease in cyclin D1 protein levels within 6 h following treatment of MCF-7 cells with the steroidal antiestrogen ICI 182780 (27). The rapid loss of cyclin D1 protein occurred as a result of transcriptional repression, by an as yet unidentified mechanism. A loss in enzymatic activity of cyclin D1-Cdk4 complexes, which preceded the decline in the proportion of actively cycling cells. The ultimate result was the accumulation of hypophosphorylated (functionally active) pRb prior to cells exiting the cell cycle (27). However, a number of other inhibitory agents such as transforming growth factor-β (28), interferon-α (29), and prostaglandins (30) act on both cyclin D1-Cdk4 and cyclin E-Cdk2 complexes. Also, cyclin E-Cdk2 has recently been shown to replace cyclin D1-Cdk4 in murine mammary gland development (31), suggesting that both cyclin-CDK complexes play a role in normal cellular proliferation in mammary epithelium.

The molecular mechanisms underpinning antiestrogen-induced cell cycle arrest are not fully elucidated nor has the physiological state in which the cells arrest been addressed. An understanding of the molecular mechanisms of antiestrogen action in breast cancer is essential to defining differences in potency and efficacy between various antiestrogens, identifying more efficacious compounds and providing deeper insight into the mechanisms of antiestrogen resistance. This article addresses two primary questions that arise from the current level of knowledge on antiestrogen action on cell cycle progression, the first being the effects of a pure estrogen antagonist, ICI 182780, on the activity and function of cyclin E-Cdk2. The second objective was to identify changes in pocket protein abundance and E2F complex formation that accompany antiestrogen-mediated growth arrest in order to provide further insight into the particular growth arrest state induced by pure estrogen antagonists.

MATERIALS AND METHODS

Cell Culture—Stock solutions of 7α-[9-(4,4,5,5,5-pentafluoro-5-(1,5,5-(1H-triazole-3,3-dioxo)-1H-triazole-3-yl)-1H-1,2,3-triazole-1-yl)]-6H-dioxolane (ICI 182780) were prepared as follows: ICI 182780 (a kind gift from Dr Alan Wakeling, Zeneca Pharmaceuticals, Alderley Park, Cheshire, United Kingdom) was dissolved in ethanol to 10–2 M and a working dilution of 10–5 M in RPMI 1640 medium was prepared from this stock immediately prior to each experiment. MCF-7 human breast cancer cells (Michigan Cancer Foundation, Detroit, MI) were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, insulin (10 μg/ml), and streptomycin (10 μg/ml). For experiments investigating the effects of ICI 182780 150-cm2 flasks were seeded with 1 × 105 cells. Cells were allowed to proliferate for 2 days until they reached approximately 50% confluence, after which ICI 182780 or vehicle was added directly to the medium. The final concentration of ethanol in the tissue culture medium was less than 0.06% and had no effect on the rate of cell proliferation. At the completion of experiments cells were harvested by brief incubation with trypsin (0.05% w/v/EDTA (0.02% w/v) as described previously (14) or as described below. Cell cycle phase distribution was determined by analytical DNA flow cytometry as described previously (32).

Immunoblot Analysis—Cells were lysed as follows: MCF-7 cell monolayers were washed twice with ice-cold phosphate-buffered saline then scraped into ice-cold lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 200 μM sodium orthovanadate, 10 μM pyrophosphate, 100 μM NaF, and 1 μM dithiothreitol). The lysates were incubated for 5 min on ice and then centrifuged (15,000 × g, 5 min, 4 °C). Equal amounts of total protein (20–40 μg) were separated by SDS-PAGE then transferred to nitrocellulose filters. Proteins were visualized using the ECL detection system (Amersham Pharmacia Biotech, Australia) after incubation (2 h at room temperature or overnight at 4 °C) with the following primary antibodies: cyclin E (C-19), p130 (C-20), p107 (C-18), E2F4 (C-20) from Santa Cruz Biotechnology Inc., Santa Cruz, CA, cyclin D1 (C-DCS-6) from Novacastro Laboratories Ltd., Newcastle-upon-Tyne, UK; p21 (catalog number C24420), Transduction Laboratories, Lexington, KY; p27 (catalog number K25020) Transduction Laboratories; or pRb (G3–245) from PharMingen, San Diego, CA. Protein quantity was quantitated by analysis of autoradiographs using densitometry (Molecular Dynamics, Sunnyvale, CA). Quantitation of protein levels by this method was linear over the range of protein concentrations and exposure times employed in these studies.

Kinase Assay—For assessment of cyclin E-associated kinase activity, MCF-7 cell monolayers were washed twice with phosphate-buffered saline then scraped into 1 ml of ice-cold lysis buffer. The lysate was placed on ice and vortexed vigorously at intervals for 60 min then centrifuged at 15,000 × g for 5 min at 4 °C and the supernatant was retained for analysis. Following the addition of protein to kinase buffer containing 1M NaCl, the cellular debris was removed by centrifugation (15,000 × g, 5 min, 4 °C). Equal amounts of total protein (20–40 μg) were separated by SDS-PAGE then transferred to nitrocellulose filters. Proteins were visualized using the ECL detection system (Amersham Pharmacia Biotech, Australia) after incubation (2 h at room temperature or overnight at 4 °C) with the following primary antibodies: cyclin E (C-19), p130 (C-20), p107 (C-18), E2F4 (C-20) from Santa Cruz Biotechnology Inc., Santa Cruz, CA, cyclin D1 (C-DCS-6) from Novacastro Laboratories Ltd., Newcastle-upon-Tyne, UK; p21 (catalog number C24420), Transduction Laboratories, Lexington, KY; p27 (catalog number K25020) Transduction Laboratories; or pRb (G3–245) from PharMingen, San Diego, CA. Protein quantity was quantitated by analysis of autoradiographs using densitometry (Molecular Dynamics, Sunnyvale, CA). Quantitation of protein levels by this method was linear over the range of protein concentrations and exposure times employed in these studies.

Detection of p21-, p27-, and p130-associated Proteins—Immunoprecipitation of p21, p27, and p130 was performed using the method described for immunoprecipitating cyclin E for kinase activity assays, except that the antibodies were chemically cross-linked to protein A-Sepharose to reduce background (33). Antibodies used were rabbit polyclonal antibodies to human p21 (Santa Cruz Biotechnology Inc., C-19), human p27 (Santa Cruz Biotechnology Inc., C-19) and human p130 (Santa Cruz Biotechnology Inc., C-20).

The immunoprecipitated proteins were resuspended in 1 × SDS sample buffer, separated by SDS-PAGE, transferred to nitrocellulose membrane, and the proteins detected using the antibodies described for Western blotting above. Confirmation of binding specificity was performed using corresponding immunogenic peptides.

Immunodepletion of p21 and p27—p21 and p27 depletion experiments followed the same protocol as in the immunoprecipitation method, except three rounds of immunoprecipitation were completed and the remaining supernatant was retained for analysis. Following three rounds of depletion, 30 μg of supernatant protein was resolved on a 12% SDS-PAGE and immunoblotted for cyclin E or cyclin D1 as described above. The depleted lysate was assayed for p21 and p27 to ensure that greater than 90% was removed.

p21 and Cyclin D1 Antisense Oligodeoxynucleotides—A 20-mer p21 antisense oligodeoxynucleotide (TCCCCAGCGGTTGTCAGACAT) (34) was synthesized (Genviva, Australia) with phosphorothioate residues at the 5′ and 3′ terminal nucleotides to minimize exonucleolytic cleavage (35). As controls, complementary (sense) and scrambled (AGCGGTTACCTGTCGATCG) oligonucleotides were manufactured. MCF-7 cells were harvested, gently syringed 4 times to minimize clumps, and 5 × 105 cells were grown in 50-mm dishes overnight. ICI 182780 was added to a final
concentration of 10 nM to the antiestrogen-treated control dishes for 2–3 h. Eight µl of Cellfectin (Life Technologies, Inc.) and oligonucleotide (800 nM) were incubated in 1 ml of serum-free RPMI 1640 for 15 min and subsequently added to the monolayer with 1 ml of RPMI 1640 medium supplemented with 10% fetal calf serum. The dishes were placed in a 37°C incubator with 5% CO2 for 90 min with intermittent mixing. The oligonucleotide/Cellfectin solution was then decanted and the monolayer washed once with RPMI 1640 (5% fetal calf serum). Ten ml of RPMI 1640 (5% fetal calf serum) was then added to each dish along with ICI 182780 at a final concentration of 10 nM to the antiestrogen-arrested control dishes. Cells were harvested for lysate and flow cytometry as described above.

A 20-mer cyclin D1 antisense oligonucleotide was designed to complement nucleotides −11 to +9 of the translation initiation codon region of the human cyclin D1 mRNA (GCTGGTGTTCCATGGCTGGG) (36) (Geneworks, Australia). Phosphorothioates were incorporated at both the 5’ and 3’ terminal nucleotides. As controls, sense and scrambled (CGGTCCGATTCGGCGTGTTG) oligonucleotides were included in all experiments. MCF-7 cells were set up as above, except 4 µl of Cellfectin was added to each dish and the final concentration of oligonucleotide was 400 nM. Cells was harvested for lysate and flow cytometry as described above.

RESULTS

ICI 182780 Inhibits Cyclin E-Cdk2 Kinase Activity—The MCF-7 model employed in this study is an established paradigm for the study of growth inhibitory effects of hormones and antiestrogens on breast cancer cell proliferation (14, 17, 27, 30–39). The presence of 10% untreated fetal bovine serum, containing endogenous steroids and growth factors, plus supplementation with 10 µg/ml insulin typifies a complex, mitogen-rich growth environment and ensures maximal exponential growth rates which enhance sensitivity to growth inhibitory agents. Our previous work on the mechanisms of antiestrogen inhibition of cell cycle progression has shown that the pure antiestrogen ICI 182780 induces growth arrest in the presence of this complex medium (27) and that subsequent addition of estradiol can reverse these growth inhibitory effects (37). Thus the model reproduces the hormonal responses typical of hormone-responsive breast cancer. Previously published data specifically identified a rapid decrease in cyclin D1 mRNA and protein levels as early events in the action of ICI 182780 (27, 38). A consequent decrease in cyclin D1-Cdk4 enzymatic activity preceded the decline in the proportion of S phase cells and was concluded to contribute to the inhibition of MCF-7 proliferation (27). The potential role of cyclin E-Cdk2 was not specifically addressed. However, total Cdk2 activity, comprising both cyclin E-Cdk2 and cyclin A-Cdk2 complexes, decreased but not until a majority of cells had arrested in G1/S (27). Therefore, to specifically assess the effects of ICI 182780 on cyclin E-Cdk2 activity, we extracted lysates from MCF-7 cells that had been treated with the drug or vehicle for various times, immunoprecipitated cyclin E and assayed in vitro kinase activity, using histone H1 as a substrate. As seen in Fig. 1, cyclin E-Cdk2 kinase activity decreased by 8 h and declined to a minimum of approximately 25% of control levels by 24 h. The decline in cyclin E-Cdk2 kinase activity preceded the reduction in the proportion of S phase cells and, therefore, in conjunction with inhibition of cyclin D1-Cdk4, likely contributed to the inhibition of cell cycle progression and cellular proliferation.

Binding of p21 to Cyclin E-Cdk2 Precedes the Inhibition of Kinase Activity and S Phase Entry—The level of cyclin E-Cdk2 complexes following antiestrogen treatment of MCF-7 cells was next investigated in order to assess whether the levels of these complexes contribute to the loss of total enzymatic activity. Immunoprecipitation experiments indicated an increase in the total number of cyclin E-Cdk2 complexes following antiestrogen treatment presumably as a result of the previously documented accumulation of cyclin E following antiestrogen treatment (37) (data not shown). In an attempt to account for the rapid reduction in cyclin E-associated kinase activity while cyclin E-Cdk2 complexes increased, immunoblot analyses of the two predominant CDK inhibitors in this cell line, p21 and p27, were performed. As seen in Fig. 2A, p21 protein levels increased at 16 h and reached maximal 4-fold levels at 48 h. p27 protein levels increased by 12 h and were about 2-fold above control at 48 h. Thus, although the total cellular levels of both inhibitors increased following ICI 182780 treatment, increases occurred several hours after the decline in the enzymatic activity of cyclin E-Cdk2 (Figs. 1 and 2), indicating that these changes in inhibitor abundance were not causative of cyclin E-Cdk2 inhibition.

To assess whether there was an increase in cyclin E-Cdk2-associated p21 or p27 prior to the decline in kinase activity, we performed a series of immunoprecipitation experiments. Immunoprecipitation of p21 and p27 followed by immunoblotting for cyclin E demonstrated a substantial increase in the amount of cyclin E-Cdk2-associated p21 and p27 (Fig. 2B). A reproducible increase in p21-associated cyclin E of 30% was detectable at 6 h and reached 4-fold by 48 h (Fig. 2B). An increase of 5-fold was seen by 48 h for p27, with the initial changes first observable at 12 h (Fig. 2B). It therefore appeared that p21-associated cyclin E-Cdk2 increased prior to a decline in kinase activity while p27-associated cyclin E-Cdk2 only increased after cyclin E-Cdk2 activity began to decrease (Figs. 1B and 2B).

Further analysis of the interaction between p21, p27, and cyclin E-Cdk2 was conducted using immunodepletion assays, in which complexes containing p21, p27, or both inhibitors were depleted from MCF-7 lysates harvested from antiestrogen-treated or control cells. The inhibitor-depleted lysate was resolved by SDS-PAGE and subsequently immunoblotted for cyclin E and Cdk2. By 6 h post-treatment approximately 30% of the total cellular cyclin E was removed when p21 was depleted (Fig. 3). This time point preceded the change in kinase activity of cyclin E-Cdk2 by 2–3 h and further supported the hypothesis that it was the initial increase in p21-associated cyclin E-Cdk2 that was responsible for the early loss of enzymatic activity.
p27 depletions, by contrast, did not remove any cyclin E until 12 h post-treatment, by which time cyclin E-Cdk2 activity had declined by approximately 50% (Fig. 1B). Immunoblots of Cdk2 following p21 or p27 immunodepletion did not show any detectable changes in total Cdk2 levels, possibly due to the high abundance of Cdk2 protein which is known to be present in excess and is not rate-limiting for cyclin E-Cdk2 activity (37).

One other mechanism of regulation of cyclin E-Cdk2 activity is via activation by the Cdk-activating kinase (CAK), which phosphorylates threonine 160 in human Cdk2 (40), resulting in a conformational change to a more active form detectable as a mobility shift on SDS-PAGE. We assessed changes in total protein levels of two of the CAK components, cyclin H and Cdk7, after ICI 182780 exposure and found no change over the time course of the experiment (data not shown). Similarly, changes in CAK activity (as measured by changes in Cdk2 phosphorylation) were not detected until a majority of cyclin E-Cdk2 activity was lost (data not shown) suggesting that changes in CAK activation of Cdk2 did not contribute to the loss of enzymatic activity.

Antisense p21 Abrogates the Antiestrogen-induced Cell Cycle Arrest—To confirm an integral role for p21 in the binding and inhibition of cyclin E-Cdk2, antisense p21 oligonucleotides were employed to deplete p21 mRNA transcripts and decrease p21 protein levels in an effort to abrogate the inhibition of cellular proliferation imposed by ICI 182780. To achieve this, MCF-7 cells that had been treated with ICI 182780 for 2 h were incubated with either p21 antisense oligonucleotide or a sense or scrambled control oligonucleotide in the presence of Cellfectin lipid vector and cells harvested for biochemical analysis or flow cytometry. Exposure to the antisense oligonucleotide resulted in a marked decrease in p21 protein levels by 24 h (Fig. 4A). At the same time after p21 antisense treatment, flow cytometric analysis (Fig. 4B) showed a clear maintenance of cell cycle arrest imposed by ICI 182780 compared with the significant decline to 26 ± 2.5% for the sense and scrambled oligonucleotide controls (p < 0.002). This suggested that reduced availability and thus inhibitory activity of p21 attenuates the cell cycle arrest imposed on MCF-7 cells as a result of antiestrogen treatment. These data support an essential role for p21 in the inhibition of cyclin E-Cdk2 complexes

![Figure 2](http://www.jbc.org/)

**Fig. 2.** Cyclin E-Cdk2-associated p21 increases prior to a change in enzymatic activity and an increase in total p21 protein levels. The experimental design is as described in the legend to Fig. 1A. Protein levels of p21 and p27 in MCF-7 cell lysates following treatment with ICI 182780 (10 nM). Total cell lysates were separated by SDS-PAGE and immunoblotted with antibodies to p21 and p27. Protein levels were determined using densitometry as described under "Materials and Methods" and plotted (B) p27 or p21 immunoprecipitates from the same lysates were resolved by SDS-PAGE and immunoblotted for cyclin E. The levels of cyclin E in the immunoprecipitation were determined by densitometry as described under "Materials and Methods."

![Figure 3](http://www.jbc.org/)

**Fig. 3.** Immunodepletion of p21 removes increasing amounts of cyclin E following ICI 182780 treatment. The experimental design is as described in the legend to Fig. 1A. MCF-7 cell lysates were immunodepleted of p21, p27, p21, and p27, or mock depleted as described under "Materials and Methods." The immunodepleted lysates were then resolved by SDS-PAGE and immunoblotted for cyclin E and Cdk2. B, the amount of cyclin E that remained following immunodepletion of p21 (□), p27 (□), or both p21 and p27 (■) was plotted as a percentage of the mock treated lanes.
ICI MCF-7 cells were treated with ICI or ethanol vehicle as described in the legend to Fig. 1 and exposed to p21 antisense (AS) oligonucleotide (800 nM), or sense (Sen) or scrambled (Scr) control oligonucleotides 2–3 h after drug or vehicle treatment. Cells were harvested for flow cytometry and biochemical analysis at 24 h. A, p21 levels following treatment with ICI (ICI) or oligonucleotides. B, % S phase cells at 24 h. S phase was measured using ethidium bromide staining followed by flow cytometry as described under “Materials and Methods.” Data presented are the mean ± S.E. of five experiments.

and therefore the arrest of breast cancer cells in response to antiestrogens.

**Decreased Expression of Cyclin D1 Facilitates Inhibition of Cyclin E-Cdk2 Activity**—p21 and p27 can be liberated from cyclin D1-Cdk4 complexes under certain conditions of growth arrest, therefore permitting these molecules to elicit a down-stream response without the need for an increase in total cellular concentration (28, 41). Since ICI 182780 decreased the levels of cyclin D1 (27), we sought to investigate the possibility that p21 re-distributed from cyclin D1-Cdk4 complexes to cyclin E-Cdk2 complexes prior to the decrease in cyclin E-Cdk2 activity. Immunoprecipitation of p21 from MCF-7 cell lysates followed by immunoblotting of cyclin D1 demonstrated a subtle but reproducible decrease in the amount of p21-cyclin D1-Cdk4 complex by 6 h which was maintained at 12 and 24 h (Fig. 5). This decrease suggested that the increase in cyclin E-Cdk2-associated p21 was a consequence of decreased cyclin D1-Cdk4-p21 complexes following antiestrogen treatment.

If the reduction in cyclin D1 protein, as a result of antiestrogen-induced transcriptional repression, resulted in the increased availability of p21 and consequent inhibition of cyclin E-Cdk2 complexes, then direct suppression of cyclin D1 gene expression should elicit a similar response. To test this, antisense cyclin D1 oligonucleotides were transfected into proliferating MCF-7 cells with sense or scrambled oligonucleotide controls. Following titration of the cyclin D1 antisense oligomer, we achieved a level of cyclin D1 protein inhibition with 400 nM oligonucleotide that approximated the decrease seen following antiestrogen treatment (Fig. 6A). At this concentration, the reduction in S phase 24 h after treatment was similar to the decline observed in antiestrogen-treated cells at the same time point (Fig. 6B). To confirm that this decrease in S phase fraction was due to a loss of cyclin E-Cdk2 activity, we performed a cyclin E kinase assay. A reduction in activity that mimicked the loss in activity seen at the same time following antiestrogen treatment was detected (Fig. 6C). These results indicated that a 30–40% reduction in cyclin D1 protein as a result of antiestrogen treatment facilitates a shift of p21 molecules into cyclin E-Cdk2 complexes with resultant inhibition of enzymatic activity and G1 to S phase progression.

**Effects of ICI 182780 on Expression and Phosphorylation of Pocket Proteins**—The sensitivity of the G1 phase CDKs (i.e., Cdk4 and Cdk2) to antiestrogen treatment (27, 41, 42) is consistent with the established sensitivity of G1 phase cells to both steroidal and non-steroidal antiestrogens (15, 17). However, the question of where in the cell cycle the cells ultimately arrest remains unresolved. Pocket protein abundance, phosphorylation state, and their ability to form complexes with different members of the E2F family of transcription factors have been proposed as signatures of cell cycle position (23, 24, 43). Consequently, we investigated these parameters following antiestrogen treatment. Initial experiments (Fig. 7) showed that the phosphorylation state of all the pocket proteins declined markedly following ICI 182780 treatment but with different kinetics. In support of previous findings, hypophosphorylated pRb was apparent within 4 h of drug treatment and by 24 h was the predominant form (27, 41, 42). Similarly, the hypophosphorylated forms of p130 (forms 1 and 2) were detected between 4 and 10 h and predominated by 24 h (Fig. 7). The changes in p130 phosphorylation states could be mimicked by the addition of antisense cyclin D1 oligonucleotides (Fig. 7B), confirming a causal effect between the initial molecular changes following ICI 182780 treatment and the changes in pocket protein phosphorylation states. In contrast to p130, a decrease in p107 phosphorylation status was not clear until 24 h. The changes in phosphorylation state were accompanied by major changes in protein abundance. Both pRb and p107 levels decreased substantially after 16 h although the effects on p107 were of greater magnitude such that it was undetectable by 48 h, while 15% of pRb remained. By contrast, p130 levels increased after 12 h to be 3–4-fold above control between 24 and 48 h (Fig. 7A).

**Antiestrogen-induced Formation of p130-E2F4 Complexes and Loss of E2F1**—The pocket protein p130 preferentially binds the E2F4 and E2F5 transcription factors (23–25) and p130-E2F4 complexes are characteristic of cell cycle arrest (24, 25). The presence of hyperphosphorylated E2F4 together with
p130-E2F4 complexes have been proposed as molecular markers of cellular quiescence (23–25, 44, 45). To elucidate whether p130-E2F4 complexes form in response to ICI 182780 treatment, we immunoprecipitated p130 from lysates harvested at various times post-antiestrogen treatment and assessed E2F4 association by immunoblotting (Fig. 8A). The presence of p130-E2F4 complexes was clearly detectable at 24 h and increased to approximately 20-fold above basal level by 48 h. Interestingly, immunoblot analysis of E2F4 in lysates from ICI 182780-treated cells indicated that total E2F4 protein levels increased as cells growth arrest. Furthermore, hyperphosphorylated forms of E2F4 accumulated between 16 and 24 h (Fig. 8B). E2F1 is negatively regulated by p130-E2F4 (23–25), raising the possibility that formation of these complexes following antiestrogen treatment may decrease expression of E2F1. Although total protein levels of E2F1 decreased, these changes occurred between 4 and 10 h after addition of ICI 182780 (Fig. 8) and preceded the formation of p130-E2F4 complexes. The detection of p130-E2F4 complexes and the slower migrating forms of E2F4 at 24 h suggests that at this time point antiestrogen-treated cells are not merely paused in G1 phase but have exited the cell cycle and are arrested in the physiologically distinct G0 state.

FIG. 6. Treatment of MCF-7 cells with cyclin D1 antisense oligonucleotides inhibits cell proliferation and decreases cyclin E-Cdk2 activity. MCF-7 cells treated with either ICI 182780 (10 nM) or ethanol vehicle for 2–3 h were incubated with cyclin D1 antisense (AS) oligonucleotide (400 nM) or a control oligonucleotide (sense (Sen) or scrambled (Scr)) and cell lysates were harvested for flow cytometry or biochemical analysis after 24 h. A, the lysates were resolved by SDS-PAGE and immunoblotted with a cyclin D1 antibody. B, percentage of cells in S phase following 24 h exposure to oligonucleotides were analyzed by ethidium bromide staining and flow cytometry. Data presented are the mean ± S.E. of four experiments. C, cyclin E immunoprecipitates were prepared as described under "Materials and Methods" and analyzed for kinase activity using histone H1 as a substrate.

FIG. 7. Changes in pocket protein abundance and phosphorylation state following treatment of MCF-7 cells with ICI 182780. MCF-7 cells were treated with ICI 182780 (10 nM) or ethanol vehicle for the described time periods and harvested as described in the legend to Fig. 1. A, equivalent quantities of total cellular protein (25 μg) were resolved by SDS-PAGE and immunoblotted using pRb, p107, or p130 antibodies. Total protein levels were determined using densitometry as described under "Materials and Methods" and plotted. B, MCF-7 cells treated with either ICI 182780 (10 nM) or ethanol vehicle for 2–3 h were incubated with cyclin D1 antisense (AS) oligonucleotide (400 nM) or a control oligonucleotide (sense (Sen) or scrambled (Scr)) and cell lysates were harvested for biochemical analysis after 24 h. These lysates were resolved by SDS-PAGE and immunoblotted with a p130 antibody.
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6–12 h following treatment, p21 was preferentially recruited to cyclin E-Cdk2 complexes.

This shift in the P21 and P27 CDK inhibitors and cyclin D1-Cdk4 and cyclin E-Cdk2 activity is mediated in full or in part through decreased cyclin D1 expression. Although a decrease in cyclin D1 would be expected to reduce cyclin E-Cdk2 activity, it is now clear that the decrease in cyclin D1 expression is not sufficient to completely account for the decrease in cyclin E-Cdk2 activity.

The conclusion that relatively minor shifts in inhibitors can influence cellular proliferation rates indicates the importance of subtle modulation in the equilibrium between inhibitors and cyclin-CDK complexes in the overall maintenance of proliferation rates.
ferentiation in vitro, in part reflecting a predominance of E2F4 in these cell types (reviewed in Ref. 24). Furthermore, recent data have shown that E2F4 is necessary for nerve growth factor-induced neurite outgrowth in differentiating PC12 cells and that increased E2F4 expression accelerates differentiation, suggesting it can promote differentiation as well as regulate cell proliferation (49). Similarly, expression of p130 in 32Dc3 myeloid cells led to morphological differentiation (50). Finally, investigation of E2F4 in Daudi cells indicated the presence of hypophosphorylated E2F4 following interferon-α-mediated growth arrest, in contrast with the hyperphosphorylated forms of E2F4 present in quiescent primary T-cells and B-cells (45, 51). Since both hypo- and hyperphosphorylated E2F4 bound p130, Thomas et al. (45) concluded that the presence of p130-E2F4 complexes was not sufficient to define quiescent cells, but that this additionally required the presence of hypophosphorylated E2F4. Interestingly hyperphosphorylated E2F4 bound preferentially to DNA, suggesting that the phosphorylation has consequences for E2F transcriptional activity (51). In cells treated with the pure antiestrogen ICI 182780, the combination of p130-E2F4 complexes, high E2F4 expression, and the presence of hyperphosphorylated E2F4 together argue that the arrested cells are in a quiescent state.

The data generated in this study underpin the model of estrogen antagonist action on the cell cycle presented in Fig. 9. Among the earliest documented responses to the pure steroid antiestrogen is decreased cyclin D1 gene expression (27, 38), with resultant decreases in cyclin D1-Cdk4 complexes and a consequent increase in free p21 and p27 that can associate with cyclin E-Cdk2. Data presented in Fig. 3 illustrate that p21 preferentially associates with cyclin E-Cdk2 at early time points, i.e. 6–12 h, as cyclin E-Cdk2 activity declines, but by 24 h both p21 and p27 are present in these complexes. The basis for the preferential early accumulation of p21 remains undefined. Increased cellular levels of p27 are apparent at 12 h when cyclin E-Cdk2 activity is half-maximally inhibited. This post-transcriptional modulation of p27 levels is likely mediated by inhibition of cyclin E-Cdk2 phosphorylation of p27, which is necessary for ubiquitination and proteasome degradation of this molecule (52). Thus the recruitment of p21 to the cyclin E-Cdk2 complex inhibits kinase activity resulting in the accumulation of a second inhibitor, p27, and almost complete inhibition of enzyme activity (Fig. 9). Precedence for such co-operation between p21 and p27 in growth inhibition has been observed during interleukin-4-mediated growth inhibition of astrocytoma cells (53). In that system interleukin-4 induced preferential accumulation of p21 in cyclin E-Cdk2 complexes at early time points and subsequent accumulation of total and cyclin E-Cdk2-bound p27, in common with the model presented here for inhibition by the estrogen antagonist (Fig. 9). More importantly, treatment with antisense p21 prevented p27 accumulation and prevented interleukin-4-mediated growth arrest, identifying induction of p21 as an early precipitating event. Increased cellular levels of p21 mRNA (data not shown) and of both p21 and p27 protein at later time points after antiestrogen treatment likely contribute to maintenance of inhibition of the cyclin E-Cdk2 complexes. This is supported by recently published data demonstrating that both antisense p21 and p27 can attenuate antiestrogen-induced growth inhibition (54). Our unpublished data confirm the effect of antisense p27, however, in support of p21 being the initiating factor in inhibition of cyclin E-Cdk2 and consequent accumulation of p27, p21 antisense treatment resulted in a decrease in total protein levels of p27 while changes in p21 protein levels were not observed following p27 antisense experiments (data not shown).2

As illustrated in Fig. 9, the subsequent loss of enzymatically active cyclin D1-Cdk4 and cyclin E-Cdk2 complexes results in decreased pocket protein phosphorylation and sequestration of E2F transcription factors by the hypophosphorylated pocket proteins. In addition, and of likely functional importance, p130 associates with E2F4 and hyperphosphorylated forms of E2F4 appear. Interestingly, inhibition of ICI 182780 mediated growth arrest with antisense oligonucleotides to p21, eliminates the formation of these hyperphosphorylated forms of E2F4 (data not shown),2 further implicating p21 in growth arrest into quiescence.

2 J. S. Carroll, O. W. J. Prall, E. A. Musgrove, and R. L. Sutherland, unpublished data.
Mechanisms of Cell Cycle Arrest by a Pure Antiestrogen

Based on this model, two primary questions remain to be answered: what are the initiating events in inhibition of cyclin D1 gene expression and what are the functional consequences of quiescence in terms of the cell’s ability to escape inhibition by estrogen antagonists? Estrogen regulation of cyclin D1 gene expression is due predominantly to transcriptional activation (37, 55). Inhibitors of protein synthesis block this increase in cyclin D1 mRNA (37, 55) and an estrogen-responsive region within the first 944 base pairs upstream of the cyclin D1 transcriptional start site does not contain a classical estrogen response element (55). These data suggest that the synthesis of intermediary proteins is essential for the estrogen effect on cyclin D1 gene transcription. More recent studies have identified a putative cAMP response element in the proximal promoter of cyclin D1 which is activated by estrogen and inhibited by antiestrogen. This requires both the AF-1 and AF-2 domains of the estrogen receptor but not direct DNA binding (56). ER appears to interact with ATF-2/c-Jun heterodimers to activate cyclin D1 gene transcription. This effect may be mediated in part by estrogen-induced c-Jun expression (56). Thus a potential mechanism of antiestrogen inhibition of ER-mediated cyclin D1 transcription is via disruption of ER-ATF-2/c-Jun complexes and/or inhibition of c-Jun expression.

This study has not addressed the potential functional consequences of antiestrogen-induced quiescence, as opposed to G1 arrest, but this may well have implications for the optimal therapeutic use of these compounds in the management of hormone-dependent cancers and antiestrogen resistance. Since a range of peptide growth factors, e.g. insulin-like growth factor-I, epidermal growth factor, and transforming growth factor-α can stimulate G1 phase progression in breast cancer cells (57) they might be expected to attenuate the growth inhibition effects of compounds that induce arrest in G1. Our preliminary data suggest that ICI 182780-arrested cells are unresponsive to these mitogenic agents (data not shown) suggesting that a major consequence of cell cycle arrest in a quiescent state could be to render cells insensitive to several growth factors that are present in high concentrations within the microenvironment of breast cancer cells (58). Thus by inhibiting estrogen-mediated gene expression and arresting cells in a quiescent state, pure estrogen antagonists may not only inhibit estrogen-induced mitogenesis but also that mediated by a spectrum of potential mitogenic growth factors, both of which would contribute significantly to the antitumor activity of these compounds.

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A Pure Estrogen Antagonist Inhibits Cyclin E-Cdk2 Activity in MCF-7 Breast Cancer Cells and Induces Accumulation of p130-E2F4 Complexes Characteristic of Quiescence

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