p27Kip1 Induces Quiescence and Growth Factor Insensitivity in Tamoxifen-treated Breast Cancer Cells

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Abstract

Tamoxifen, a selective estrogen-receptor modulator, is effective in the treatment and prevention of breast cancer, but therapeutic resistance is common. Pure steroidal antiestrogens are efficacious in tamoxifen-resistant disease and, unlike tamoxifen, arrest cells in a state of quiescence from which they cannot reenter the cell cycle after growth factor stimulation. We now show that in hydroxytamoxifen-treated cells, transduction of the cell cycle inhibitor p27Kip1 induces quiescence and insensitivity to growth stimulation by insulin/insulin-like growth factor I and epidermal growth factor transforming growth factor alpha (3–5). Structurally distinct steroidal antiestrogens, of which ICI 182780 are indicative of arrest in G0 (10). The ICI 182780-induced quiescent state is associated with insensitivity to mitogenic growth factors (13). In contrast, SERM treatment is less effective in the presence of growth factors, raising the possibility of a different state of growth arrest (14, 15). Recruitment of the corepressors N-CoR and SMRT to the ER has been suggested as one potential mechanism by which tamoxifen inhibits cell proliferation (16). However, it is unclear how corepressor utilization by tamoxifen might contribute to growth factor sensitivity and modulation of antiestrogen sensitivity. It is also unclear whether corepressor utilization acts in concert with other events, such as p27Kip1 accumulation, or whether these are distinct pathways used by different classes of antiestrogens.

Introduction

Antiestrogens, including SERMs (e.g., tamoxifen) and pure estrogen antagonists, are potent inhibitors of proliferation in ER-positive breast cancer cells, where they antagonize the mitogenic effects of estrogen (1). Tamoxifen is the endocrine therapy of choice for the treatment of hormone-sensitive breast cancer and has also shown efficacy in some prevention trials (2). However, the effectiveness of tamoxifen is restricted by the frequent development of cellular resistance (2, 3). The mechanisms underlying tamoxifen resistance are not well understood (1–4), but there is increasing evidence that up-regulation of tyrosine kinase receptors, particularly the erbB receptors, and their signaling pathways are important in endocrine resistance (3–5). Structurally distinct steroidal antiestrogens, of which ICI 182780 is one example, have been developed that lack agonist activity and have greater long-term effectiveness than tamoxifen in inhibiting breast cancer cell growth in vitro and in vivo (6, 7). Because a proportion of tamoxifen-resistant breast cancers retain sensitivity to steroidal antiestrogens (7), differences in the mechanisms of action of the two classes of antiestrogen could potentially include novel pathways that contribute to resistance.

The molecular mechanisms of action of ICI 182780 as a growth inhibitory agent in vitro have recently been clarified. Treatment of estrogen-responsive MCF-7 breast cancer cells induces down-regulation of the G1-specific cyclin, cyclin D1 (8), as a direct consequence of inhibition of expression of the proto-oncogene c-Myc (9). This initiates a cascade of molecular events, including the redistribution of the CDK inhibitor p21WAF1/CIP1 from cyclin D1-Cdk4 complexes to cyclin E-Cdk2 complexes, inhibition of cyclin E-Cdk2 activity, and the consequent accumulation of the CDK inhibitor p27Kip1 (10, 11). A distinguishing feature of ICI 182780-mediated growth arrest is induction of quiescence (10). Quiescence (G0) is a physiological state that is distinct from G1 in terms of responses such as the time required for cell cycle reentry after mitogenic stimulation and the ability to initiate DNA synthesis. Molecular differences between G0 and G1 include hyperphosphorylation of the transcription factor E2F4 and its association with the pocket protein p130 during G0 to form p130-E2F4 complexes that are thought to mediate much of the transcriptional repression of genes specifically down-regulated in G0 (12). Consequently, the accumulation of p130 and its association with E2F4 after treatment with ICI 182870 are indicative of arrest in G0 (10). The ICI 182870-induced quiescent state is associated with insensitivity to mitogenic growth factors (13). In contrast, SERM treatment is less effective in the presence of growth factors, raising the possibility of a different state of growth arrest (14, 15). Recruitment of the corepressors N-CoR and SMRT to the ER has been suggested as one potential mechanism by which tamoxifen inhibits cell proliferation (16). However, it is unclear how corepressor utilization by tamoxifen might contribute to growth factor sensitivity and modulation of antiestrogen sensitivity. It is also unclear whether corepressor utilization acts in concert with other events, such as p27Kip1 accumulation, or whether these are distinct pathways used by different classes of antiestrogens. Because insights into the molecular mechanisms whereby SERMs and pure antiestrogens induce growth arrest may shed light on cellular pathways that contribute to antiestrogen resistance, we investigated the growth arrest states induced by SERMs and pure antiestrogens and the mechanisms for their differential sensitivity to growth factor-mediated cell cycle reentry.

Materials and Methods

Antiestrogens. Stock solutions of antiestrogens were prepared as follows: ICI 182870 (a kind gift from Dr. Alan Wakeling, AstraZeneca Pharmaceuticals, Alderley Park, Cheshire, United Kingdom), hydroxytamoxifen (from Dr. A. Wakeling), CI 628 (from Dr. E. Eislager, Warner-Lambert Parke Davis, MI, ICI 164384 (from Dr. A. Wakeling), and RU 58668 (a kind gift from Dr. P. Van de Velde, Hoechst Marion Roussel, 93235 Romainville, France) were dissolved in ethanol to 0.01 M and a working dilution in RPMI 1640 medium.
was prepared from this stock immediately before each experiment. Hydroxy-
clomiphene (from Merrell-Dow Pharmaceuticals, OH) stock solutions were
prepared as 0.01 m stocks in DMSO and working dilutions were prepared in
RPMI 1640 on the day of use.

**TAT-p27 Protein.** The TAT-p27 construct was a kind gift from Dr. Steven
Dowdy (Washington University School of Medicine, St. Louis, MO). Tat-
p27Kip1 protein was produced as described (17), except protein was dialyzed
exhaustively and concentrated by acetone precipitation.

**Cell Culture.** The MCF-7 and MDA-MB-231 cell lines were obtained
from EG & G Mason Research Institute (Worcester, MA). These cell lines
were cultured and DNA flow cytometry was performed as described previ-
ously (10). Antiestrogens were added to cells in exponential growth phase and
were cultured and DNA flow cytometry was performed as described previ-
ously (10). Polyclonal antibodies raised against human p130 (C-20),
SMRT (C-19), N-CoR (C-19), SRC-1 (M-341), and pCIP/SRC-3 (M-397)
were obtained from Santa Cruz Biotechnology Inc.

**Chromatin Immunoprecipitation.** Cells were fixed with formaldehyde
(1% final concentration) and treated as described previously (16). Briefly,
the soluble chromatin was extracted, precleared with salmon sperm DNA and
protein A-Sepharose beads, then immunoprecipitated overnight at 4°C
using the SRC-1 and p/CIP antibodies described above. DNA was extracted from
the immunoprecipitates and purified before PCR. The following primers were
used for PCR of the pS2 promoter region (16): forward, 5'-CTATGAACT-
ACTTCTGAGTGAGT-3' and reverse, 5'-CCGATTATATGGGCAG-GCTCTG-3'.

**Indirect Immunofluorescence.** MCF-7 cells were initially grown on acid-
coverslips in RPMI/10% FCS, then serum-starved in the presence of
hydroxytamoxifen, and subsequently treated with insulin (24 μg/ml) and gentamicin
(10 μg/ml). This medium was changed daily for the length of the experiment.
Hydroxytamoxifen or hydroxyclomiphene was added for a further 24 h. In

**Results**

**Pure Antiestrogens and SERMs Induce Different Growth Arrest States.** Our previous observation that ICI 182780 arrests MCF-7
human breast cancer cells in quiescence (10) raised the question of
whether this response was also induced by other classes of antiestro-
gens. We compared the responses of MCF-7 cells treated with three
SERMs [hydroxytamoxifen (the active metabolite of tamoxifen), hy-
droxyclomiphene, and CI 628] and three pure steroidal antiestrogens
(ICI 182780, ICI 164384, and RU 58668). The pure steroidal antiestrogens
decreased the S-phase fraction from 37% to 6–8%, but the
SERMs were significantly less effective even at high concentrations,
decreasing the S-phase fraction to 22–24% (Fig. 1A). p130-E2F4
association, a marker of quiescence, was apparent in cells treated with
pure antiestrogens, but not SERMs (Fig 1B), indicating that the
SERMs did not arrest breast cancer cells in quiescence. No changes were
observed in the ER-negative cell line, MDA-MB-231, after treatment with
either SERMs or pure antiestrogens (Fig. 1B and data not shown), confirming that these responses are ER-mediated.

We reasoned that if distinct growth arrest states were induced by the
different classes of antiestrogens, the ability of cells to reenter the
cell cycle after mitogen exposure might be differentially altered. The proportion of cells that responded to mitogenic stimulation from an
antiestrogen-arrested state in the first 24 h after insulin stimulation
was only 5–8% after pretreatment with pure antiestrogens, but ~30% af-
after treatment with the SERMs (Fig. 1C). Cells stimulated with
IGF-I, EGF, or TGFα behaved in a similar manner, demonstrating
clear differences in the ability of cells to reenter the cell cycle after arrest with the two classes of antiestrogen and consistent with the conclusion that they induce different growth arrest states.

Transduction of Hydroxytamoxifen-treated Cells with TAT-p27 Mimics ICI 182780-mediated Growth Arrest. An increase in p27Kip1 plays an essential role in maintaining growth arrest of MCF-7 cells after ICI 182780 treatment (10, 11). Because p27Kip1 has been implicated in the maintenance of quiescence (18, 19), differential regulation of p27Kip1 is a potential mechanism through which different antiestrogens might induce different states of growth arrest. Consistent with this hypothesis, 48-h treatment with the pure antiestrogens increased the level of p27Kip1 2–3-fold, whereas SERM treatment had little effect (Fig. 2A).

To determine whether the increased p27Kip1 abundance observed after treatment with pure antiestrogens was a pivotal event mediating entry into quiescence and consequent mitogenic insensitivity, we assessed the effects of introduction of exogenous p27Kip1 via TAT-mediated protein transduction (17) on the potency of hydroxytamoxifen. In serum-starved cells treated with hydroxytamoxifen or TAT-p27 alone, little or no p130-E2F4 association was seen (Fig. 2B). However, the combination led to significant p130-E2F4 association, as seen after ICI 182780 treatment (Fig. 2B). Furthermore, exogenous p27Kip1 also decreased sensitivity to mitogenic stimulation, because the addition of TAT-p27 decreased the percentage of insulin-stimulated cells that escaped hydroxytamoxifen-mediated growth arrest from 30% to 15% (Fig. 2C). Thus, the transduction of TAT-p27 into hydroxytamoxifen-treated cells elicited a response similar to that induced by ICI 182780, i.e., growth arrest in quiescence and decreased sensitivity to mitogens.

Pure Antiestrogen and SERM Effects on ERα Abundance and Association with the Corepressors N-CoR and SMRT. Previous work identified the significant down-regulation of ERα by ICI 182780 (20) and the recruitment of the corepressors N-CoR and SMRT by tamoxifen (16) as potential mechanisms by which different antiestrogens can mediate growth arrest. ERα decreased after treatment with pure antiestrogens, but not after treatment with any of the SERMs, which, instead, increased ERα levels (Fig. 3A). In addition, various mitogenic signaling pathways can negatively regulate the ability of N-CoR and SMRT to associate with ERα (21). Therefore, we investigated the role of corepressor association with ERα in the mitogen sensitivity of cells treated with different SERMs. A significant increase in SMRT and N-CoR association with ERα was apparent after
SERM treatment but the pure antiestrogens did not induce detectable association of ERα with either of the corepressors (Fig. 3A). Thus, the previously described mechanisms are characteristic of these classes of antiestrogens.

We next investigated whether corepressor association with ERα could be regulated by growth factors that reinitiate cell cycle progression in SERM-treated cells. MCF-7 cells were serum-starved in the presence of hydroxytamoxifen or hydroxyclomiphene and then stimulated with insulin. Corepressor-ERα association was markedly decreased after insulin stimulation, and, conversely, the association of two coactivators, SRC-1 and p/CIP, was increased (Fig. 3B). To determine whether this exchange between corepressors and coactivators resulted in changes in ERα-mediated transcription, we measured coactivator occupancy of a known estrogen-regulated gene promoter, pS2, using chromatin immunoprecipitation. In cells serum-starved in the presence of hydroxytamoxifen and then restimulated with insulin, SRC-1 associated with the pS2 promoter within 30 min of insulin stimulation and this increased after 1 h (Fig. 3C). Interestingly, p/CIP association with the same site in the pS2 promoter increased after 30 min, but returned to baseline by 1 h (Fig. 3C). These data indicate that mitogen stimulation alters coactivator recruitment and hence ERα activity at the promoters of estrogen-regulated genes.

We next investigated whether subcellular relocalization might be a cause of modulation of ER binding and transcriptional inhibition by the corepressors N-CoR and SMRT. Indirect immunofluorescence of cells serum-starved in the presence of hydroxytamoxifen revealed nuclear localization of both N-CoR and SMRT (Fig. 4A). However, after insulin stimulation, cytoplasmic distribution of both N-CoR and SMRT increased substantially (Fig. 4A). To further define the signaling pathways used by insulin to regulate the corepressor-ER complex, we used specific chemical inhibitors of either the mitogen-activated protein kinase or phosphatidylinositol 3'-kinase pathways: U0126 and Wortmannin, respectively. MEK inhibition before insulin stimulation inhibited the cytoplasmic relocalization of N-CoR (Fig. 4B). Similar results were obtained for SMRT (data not shown). In marked contrast, inhibition of phosphatidylinositol 3'-kinase with Wortmannin did not influence the insulin-mediated redistribution of N-CoR or SMRT to the cytoplasm (Fig. 4B and data not shown). Thus, the cytoplasmic redistribution of N-CoR and SMRT appears to be mediated by signaling through MEK.

**Discussion**

In these experiments, we have shown that not all antiestrogens induce the same growth arrest state: all three pure antiestrogens arrested MCF-7 cells in quiescence, but the SERMs did not. In addition, whereas SERM-treated cells responded to mitogenic stimulation, cells treated with pure antiestrogens were essentially insensitive. These data raised the question of the molecular mechanisms of growth arrest. One candidate mechanism that could potentially account for this disparity was modulation of the CDK inhibitor p27Kip1 which is essential for long-term growth arrest by ICI 182780 (10, 11), and is induced in MCF-7 xenographs after long-term administration of RU 58668 concomitant with a significant decrease in tumor volume (22). Despite this, accumulation of p27Kip1 did not appear to be a major mechanism of growth regulation by the SERMs. The presence of hydroxytamoxifen alone or p27Kip1 alone did not cause quiescence, suggesting that although either can inhibit cell cycle progression, neither is sufficient to induce quiescence. Significantly, however, the addition of exogenous p27Kip1 to hydroxytamoxifen-treated cells resulted in growth arrest in quiescence as indicated by p130-E2F4 association, and conferred mitogen insensitivity.

The absence of p130-E2F4 complexes in SERM-treated cells indicated that they were arrested in G1 rather than in G0, consistent with the observation that after treatment with any of the three SERMs, a third of the cell population reentered the cell cycle from an arrested state in the first 24 h of mitogen stimulation. These restimulated cells apparently maintained key priming events required for DNA synthesis and cell division that were not present in cells treated with pure antiestrogens, because in contrast with the response of SERM-treated cells, very few of the cells that were treated with the pure antiestrogens could reenter the cell cycle after insulin stimulation. This lack of response did not simply reflect an elongated period of time required for exit from G0 into the cell cycle, because insulin stimulation for >48 h did not increase the percentage of cells synthesizing DNA (data...
not shown). These data support the hypothesis that quiescence requires initial cell cycle arrest, followed by a secondary event (likely p27^Kip1 induction) that causes the transition from G1 into G0. The effects of hydroxytamoxifen treatment and subsequent p27^Kip1 addition are reminiscent of ICI 182780-mediated growth arrest, which initially involves inactivation of cyclin E-Cdk2, followed by accumulation of p27^Kip1 and subsequent growth arrest in quiescence (10). These data emphasize the importance of the accumulation of p27^Kip1 in induction of quiescence after treatment with the pure antiestrogens, consistent with previous observations in other cell types that implicate p27^Kip1 in the induction and maintenance of a quiescent state (18, 19).

A second mechanism of growth regulation investigated was mod-ulation of ERα, which was strongly down-regulated by pure antiestrogens but not by the SERMs. The decline in ERα protein levels after pure antiestrogen treatment likely inhibits both classic ER-mediated transcription and growth factor-mediated activation of the ER complex and may be the fundamental reason why the pure antiestrogens are solely estrogen antagonists. Although the SERMs did not decrease ERα protein levels, their did recruit the corepressors N-CoR and SMRT to the ER. Evidence including the correlation between tamoxifen resistance and decreased levels of N-CoR and SMRT (23) or increased levels of the coactivator AIB1 (24) indicates that this is likely to be an important in vivo growth inhibition mechanism for SERMs.

The ability of mitogens to attenuate the effects of tamoxifen and structurally similar compounds was identified more than a decade ago (14, 15). We now provide a mechanistic explanation for these observa-tions, where recruitment of corepressors to the ER is impaired by the presence of growth factors that induce redistribution of the corepressors to the cytoplasm. The MEK signaling pathway increases SMRT phosphorylation, which induces subcellular relocalization of the corepressor and, therefore, decreases transcriptional inhibitory activity (25). The dissociation of the corepressors from the ER may destabilize the scaffolding of the multisubunit transcriptional repres-sor complex containing SMRT, Sin3A, and histone deacetylases (26), thereby impairing ER-mediated repression. We show that in the presence of mitogenic growth factors the association of the corepressors N-CoR and SMRT with tamoxifen-bound ER is greatly diminished and replaced by association with the transcriptionally active coregulators SRC-1 and p/CIP. Furthermore, the presence of SRC-1 and p/CIP-associated ER correlated with the localization of the activators to the promoter of the ER-regulated gene pS2. Thus, coac-tivator association with tamoxifen-bound ER may be regulated by mitogen-mediated subcellular redistribution of corepressors and consequently the ability of tamoxifen to growth-arrest MCF-7 cells may be greatly influenced by the presence of mitogens that activate signaling pathways capable of regulating corepressor association with ER-containing complexes. In summary, we have shown transduction of p27^Kip1 into hydroxymethoxifen-treated cells mimics the effects of pure antiestrogens. We also show that growth factor-induced reversal of tamoxifen arrest is accompanied by modulation of ERα-cofactor interactions. Overall, these data suggest that therapeutic strategies aimed at elevating p27^Kip1 or inhibiting MEK should be tested for their ability to reverse antiestrogen resistance in preclinical models and in the clinic.

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References

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