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Raloxifene Inhibits Growth Of RT4 Urothelial Carcinoma Cells Via Estrogen Receptor-Dependent Induction Of Apoptosis And Inhibition Of Proliferation

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Abstract

Bladder cancer is the fifth most common type of cancer in the United States, with over 70,000 new cases diagnosed each year. Treatment often involves invasive surgical therapies, as chemotherapy alone is often ineffective and associated with high recurrence rates. Identification of estrogen receptor- β (ER β) in up to 75% of urinary tumors raises the question of whether this receptor could be targeted to effectively treat bladder cancer. In this study, a panel of five bladder cancer cell lines representing a variety of disease stage and grades were treated with the antiestrogens 4-hydroxytamoxifen, raloxifene or the pure antagonist ICI 182,780. All cell lines were ER β -positive, while only a few expressed ER α . Notably, all but the TCCSUP cell line were growth inhibited 20-100% by at least two antiestrogens. Using RT4 cells, we demonstrate that growth inhibition by raloxifene is ER-dependent and either ER α or ER β can mediate this response. Activation of caspase-3 and its effector PARP demonstrate that raloxifene-induced growth inhibition is in part the result of increased apoptosis; this PARP cleavage was ER-dependent. Moreover, changes in the expression of cell cycle genes indicate that cell proliferation is also affected. Specifically, raloxifene treatment results in the stabilization of p27 protein, likely via the downregulation of SKP2. Expression of the negative cell cycle regulator B-cell Translocation Gene 2 (BTG2) is also increased, while cyclin D1 transcription is reduced. These results indicate that antiestrogens may be useful targets in the treatment of bladder cancer by targeting ER and inhibiting growth via multiple mechanisms.

Keywords

Estrogen receptor; estrogen receptor modulator; bladder cancer; apoptosis; cell cycle

Introduction

Bladder cancer is the fifth most common type of cancer in the United States, with over 70,000 new diagnoses reported annually [1]. Nearly 90% of these cases are urothelial cell carcinomas (UCC), which arise in the epithelial lining of the bladder wall. Despite established treatments like transurethral resection and intravesical therapy, recurrences are

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frequent and range from 30-80%, depending on initial tumor stage and grade [2,3]. This high recurrence rate coupled with extensive follow-up care makes bladder cancer the most expensive malignancy from diagnosis until death [4]; hence, there is an urgent need to develop new effective therapies for this disease.

The estrogen receptors ER α and ER β are transcription factors that modulate diverse cellular processes such as proliferation, differentiation, and apoptosis [5]. Encoded by unique genes, these receptors are structurally similar with each containing an N-terminal activation function-1 (AF-1) and a ligand-dependent AF-2 located near the C-terminus. In ER α the AF-1 and AF-2 function independently but can synergize to produce maximal transcriptional activity [6]. Conversely, the AF-1 of ER β contains a repression domain that suppresses transcription responses [7]. These differences are thought to contribute to the receptors' oft opposing functional outcomes. While ER α can promote the proliferation of breast epithelial and other cell types, the effects of ER β are markedly anti-proliferative and pro-apoptotic [7,8]. The latter receptor additionally regulates cell motility, negatively impacting invasion and promoting cell adhesion [8]. In spite of these anti-tumor-like activities, expression of ER β does not always correlate with improved disease outcomes. For instance, in non-small cell lung cancer high levels of ER β predict reduced survival in patients with advanced disease [9]. Moreover, estrogen-stimulated proliferation of these cells is ER β -dependent [10]. Thus, like ER α , ER β can also mediate proliferation, albeit in a cell type-specific manner.

Normal bladder urothelium predominantly expresses ER β , and this receptor is found in up to 75% of UCCs [11-13]. Interestingly, ER β is more often detected in tumors of advanced stage and grade [11,12], though high levels of ER β expression in non-invasive, low grade tumors predict better recurrence-free survival [14]. In contrast, ER α has been reported in less than 30% of UCC samples [11,12,15]. Given the prevalence of ER α and ER β in UCC and their ability to impact proliferation in other cells types, it was hypothesized that these receptors represent novel targets for the treatment of bladder cancer.

Antiestrogens such as the selective estrogen receptor modulator (SERM) tamoxifen are currently used clinically to treat ER α -positive breast cancers. These compounds compete with the receptors' cognate ligands to antagonize activity. Yet despite their ability to bind to ERs, antiestrogen effects, namely inhibition of proliferation and induction of apoptosis, can occur through both ER-dependent and ER-independent means [16,17]. For example, tamoxifen produces a G1 arrest in breast cancer cells through the ER-mediated downregulation of cyclin D1, but this drug can also inhibit the proliferation of a number of ER-negative malignancies via the modulation of mitogen-activated protein kinase and protein kinase C signaling cascades [18-21]. Moreover, antiestrogens do not require ER to stimulate caspase-dependent apoptosis at high doses [22]. Therefore, many cell types with varying levels of ER expression may be responsive to antiestrogen treatment.

Early reports exploring the use of SERMs in bladder cancer showed that tamoxifen enhanced UCC sensitivity to standard chemotherapeutics *in vitro* [23], while a more recent study found that tamoxifen and raloxifene could inhibit the growth of the invasive UCC cell line 5637 [24]. These reports did not examine the ER-dependency of these responses, nor was the mechanism of antiestrogen action determined. In the present study, we screened a panel of UCC cell lines for ER expression and examined whether tamoxifen, raloxifene or the pure antiestrogen ICI 182,780 (ICI) could inhibit their growth. As the majority of bladder cancers are diagnosed as low grade, non-invasive tumors [1,25], the representative cell line RT4 was used to further characterize the antiestrogen response, including its dependency on ER and its ability to affect apoptosis and cell cycle progression.

Materials & Methods

Cell Lines and Culturing Conditions

The 5637, MCF-7, PC-3, RT4, T24, and TCCSUP cell lines were obtained from the American Type Culture Collection (ATCC) and cultured according to ATCC recommended conditions for each cell line. TSU-Pr1 cells, a gift from Dr. Tim Thompson [12], were cultured in RPMI media containing 10% fetal bovine serum (FBS). The UM-UC-9 and UM-UC-14 cell lines were the generous gift of Dr. David McConkey (University of Texas-MD Anderson Cancer Center) and were cultured in Minimum Essential Media (MEM) containing 10% FBS, 2% MEM vitamins, 1% sodium pyruvate, and 1% non-essential amino acids. The SVHUC line was a kind gift from Dr. J.T. Hsieh (University of Texas-Southwestern) and was cultured in T-medium with 5% FBS. For all experiments, cells were plated in growth media containing 10% FBS to improve adherence. The following day cells were washed with phosphate-buffered saline (PBS), and unless otherwise indicated, experiments were carried out in phenol red-free Dulbecco's Modified Eagle (DME) media containing 1% charcoal-stripped FBS (sFBS) and 1X GlutaMAX I (Invitrogen).

ER ligands

The ER ligands 17 β -estradiol (E2), 4-hydroxytamoxifen (4HT) and raloxifene (Ral) were obtained from Sigma Aldrich. ICI 182,780 (ICI) was purchased from Tocris.

Cell Proliferation Assays

For MTS proliferation assays, cells were plated at low density and ligands applied the following day in growth media containing 1% sFBS. Six days later, CellTiter 96 Aqueous One Solution (Promega) was added according to manufacturer's instructions. Cells were incubated for 2 hours at 37°C, and absorbance was measured at 450nm. Cell counts were determined for RT4 and TSU-Pr1 cells treated with ligand at the indicated concentrations in growth media containing 1% sFBS. Cells were incubated for eight days, with fresh media applied on days four and six. On day eight, cells were harvested and counted using a Beckman Coulter Z1 Particle Counter. For cell number determinations by crystal violet, cells were plated as noted above and incubated for 24 hours in DME media prior to ligand addition. Following ligand treatment and culture for 1-5 days, cells were washed with PBS, incubated with a solution of 1% crystal violet (w/v) in 10% ethanol for 10 min at RT and then extensively washed with PBS. Plates were dried, and stain was subsequently solubilized in 10% acetic acid. Absorbance was determined at 595 nm. Assays to determine ³H-thymidine incorporation were carried out in parallel with crystal violet assays. RT4 cells were treated with ligand for 18 hours at which time ³H-thymidine was added. Six hours later (24 hours total ligand exposure) cells were fixed in a 3:1 solution of methanol and acetic acid. Excess ³H-thymidine was removed with 5% trichloroacetic acid, and cells were lysed in 1 N NaOH. Lysates were neutralized in Biodegradable Counting Scintillant (Amersham) containing HCl, and radioactivity was counted using a Beckman LS 6500 Scintillation Counter (Beckman).

RT-qPCR

Total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's recommendations. First strand cDNA synthesis was carried out using qScript cDNA SuperMix (Quanta) and subsequent real time PCR was performed in an ABI 7500 Real-Time PCR System using low ROX forms of PerfeCTA SYBR Green FastMix or PerfeCTA FastMix II (both from Quanta) for SYBR Green or Taqman probe detection, respectively. Following manufacturer's instructions, qPCR reactions containing cDNA, FastMix, and primers or primers/probe were denatured at 95°C for 1 min and then cycled according to a 2-

step protocol for 40 cycles (5 sec at 95°C, 45 sec at 60°C). Target gene expression was calculated using the $\Delta\Delta C_t$ method and normalized to levels of 18S RNA. SYBR Green was used to detect amplification using previously reported primers of ER α [12], ER β [12] and cyclin D1 [26] cDNAs. Taqman gene expression assays (Applied Biosystems) with pre-mixed primer/probe were used to detect amplification of BTG2, p27, and SKP2 cDNAs. Taqman chemistry also was used to quantify levels of 18S RNA (Applied Biosystems).

Western Blotting

For detection of ER α , ER β and p27, western blotting was carried out as previously described [27]. For efficient recovery of PARP and caspase-3, cells were collected in TEN buffer [40mM Tris, pH 8, 1mM EDTA and 150mM NaCl], resuspended in PARP extraction buffer [62.5 mM Tris, pH 6.8, 6M Urea, 10% glycerol, 2% SDS, 0.003% bromophenol blue, and 5% 2-mercaptoethanol] and sonicated for 1 min at maximum amplitude in an S-4000 Bath Ultrasonic Liquid Processor (Misonix). Samples were stored overnight at -80°C and then heated to 75°C for 15 min. Protein separation was carried out on equivolume samples, and blotting continued as described above. Primary antibodies used include ER α (HC-20; Santa Cruz), ER β (H-150; Santa Cruz), p27 (C-19; Santa Cruz), PARP (Anti-PARP; Roche), caspase-3 (3G2; Cell Signaling), actin (Clone C4; Millipore) and β -tubulin (Clone AA2; Millipore).

RNA Interference

RT4 cells were transfected with 50 pmol siGENOME SMARTpool siRNA (Dharmacon: Non-targeting siRNA #3, D001210-03; ESR1, M-003401-04; or ESR2, M-003402-04) using Lipofectamine RNAiMAX (Invitrogen) for each well of a 6-well multiplate. Following overnight incubation, media was replaced with DME containing 1% sFBS, 1X GlutaMAX I and ligand treatments.

Cell Death ELISA

RT4 cells were treated with raloxifene and/or the caspase inhibitor Z-VAD-FMK (Promega) in growth media containing 5% FBS and harvested 24 hours later. Apoptosis was determined using a colorimetric Cell Death Detection ELISA kit (Roche), following the manufacturer's instructions.

Results

The extent to which antiestrogens inhibit the growth of bladder cancer cells was tested in five UCC cell lines representing non-muscle invasive, invasive, and metastatic disease. Proliferation was determined by MTS assay following six days of ligand treatment. As expected, 5637 cells, which were previously shown to be growth inhibited by 4HT [24], exhibited reduced proliferation in the presence of this ligand as well as the pure antiestrogen ICI (Fig. 1A). Similarly, a 30-45% growth inhibition was detected in T24 cells treated with these compounds, while raloxifene had no effect on either of these cell lines. In contrast, raloxifene produced the greatest effect in RT4 cells, inhibiting growth by 76% over vehicle treatment. Exposure to 4HT also limited proliferation of this non-muscle invasive cell line by 47%. The strongest antiestrogen response occurred in TSU-Pr1 cells, where robust growth inhibition was observed for cells treated with either 4HT or raloxifene. However, these cells failed to respond to ICI treatment in these MTS assays. Only the metastatic TCCSUP cell line did not respond to any of the ligand treatments, suggesting that the growth inhibitory effects of antiestrogens are widespread across multiple bladder cancer cell lines.

The MTS assay, which infers proliferation from metabolic activity, can conceal growth responses to treatments that alter mitochondrial density or function [28,29]. Consequently, to confirm the growth inhibitory effect of antiestrogens in bladder cancer cells, a Coulter counter was used to directly determine the number of RT4 cells following ligand treatment (Fig. 1B). As above, reduced cell numbers for cultures treated with either 4HT or raloxifene were observed; however, SERM-induced growth inhibition appeared stronger by cell count compared to MTS assay. Additionally, cell counts revealed a significant 54% decrease in the number of RT4 cells upon ICI treatment. A similar outcome was determined for TSU-Pr1 cells wherein Coulter counter measurements revealed an 85% decrease in cell number induced by ICI treatment while none was detected by MTS assay. These results suggest that antiestrogens can alter the metabolism of UCC cell lines, which is consistent with the increased mitochondrial activity observed in ICI-treated MCF-7 cells [29], and indicates that the MTS assay underestimates the capacity of antiestrogens to inhibit UCC growth.

Antiestrogens exert their effects in part by competing with cognate ligands to bind and disrupt ER function. To assess ER expression in cell models of bladder cancer, seven UCC cell lines were screened for both ER α and ER β mRNA expression. These included 5637, RT4, T24, TCCSUP, TSU-Pr1, UM-UC-9, and UM-UC-14 cells. Data indicate that compared to ER α -positive MCF-7 breast cancer cells, only 5637 and RT4 cells were positive for ER α transcript, with levels ~1% of those detected for MCF-7 cells (Fig. 2A). Low levels of ER α mRNA were also detected for SVHUC, a normal urothelial cell line immortalized by large T antigen. In contrast, compared to the ER β -positive PC-3 prostate cancer cell line [30,31], ER β transcript was found in all UCC cell lines tested as well as the non-malignant SVHUC cells (Fig. 2C). These results indicate that most bladder cancer cell lines primarily express ER β while few are positive for ER α —findings analogous to ER expression patterns in human tumors, particularly those with increased tumor stage and grade [11,12]. Notably, TCCSUP cells, which did not respond to antiestrogen treatment, expressed some of the lowest levels of ER β mRNA.

Although a considerable number of cell models exist for advanced bladder cancer, the majority of UCCs are diagnosed as low grade, non-muscle invasive tumors [1,25]. Recent genetic analyses identified RT4 as a clinically relevant UCC cell line, representative of low grade, non-muscle invasive disease [32]. Moreover, the RT4 gene expression profile correlates with long-term patient survivorship, a hallmark of low grade urothelial carcinomas [32,33]. Consequently, we decided to use this cell line as a representative model of bladder cancer to explore the mechanism of the aforementioned antiestrogen response and its potential dependency on ER. Prior to examining possible mechanisms of antiestrogen-induced growth inhibition, immunoblots were used to confirm ER protein expression in RT4 cells. Compared to the MCF-7 positive control, only a small amount of ER α protein was detected in RT4 cells, a result consistent with the low level of ER α transcript found in this cell line (Fig. 2B). In contrast, ER β protein was readily detected, with levels even greater than those of the ER β positive control PC-3 (Fig. 2D). Thus, like many bladder tumors, RT4 cells predominantly express ER β . However, identification of ER α and ER β protein in this cell line provided an opportunity to assess whether both receptors could contribute to the UCC antiestrogen response.

To determine if antiestrogen-induced growth inhibition of bladder cancer cells was ER-dependent, RT4 cells were transiently transfected with ER-targeted siRNA and subsequently treated with raloxifene. Since RT4 cells express both estrogen receptors, ER dependency was first established by removing ER α and ER β simultaneously. Analysis of receptor expression following RNA interference indicated a 60% reduction in ER α and ER β transcripts while receptor proteins were barely detectable (Fig. 3B,C). Raloxifene treatment in cells transfected with non-targeting control siRNA induced a 70% reduction in RT4 cell

number (Fig. 3A). However, this response was completely abrogated in cells depleted of ER α and ER β , indicating that raloxifene-induced growth inhibition of RT4 cells is ER-dependent. Notably, in the absence of ligand, cell proliferation was unperturbed by loss of ER, with cells depleted of ER α and ER β growing at a rate 1.4 ± 0.3 times ($p=0.28$) that of cells expressing these receptors.

Next, to determine which ER subtype facilitated the antiestrogen growth response, RT4 cells were transfected with siRNA targeting each ER independently. Subsequent raloxifene treatment revealed growth inhibition under all knockdown conditions, reducing cell number by 55%, 70% and 60% in siControl-, siER α - and siER β -transfected cells, respectively (Fig. 4A). These data, in combination with that of the ER double knockdown, indicate that both ER α and ER β have the potential to mediate the antiestrogen response as both must be depleted to block the raloxifene-induced growth inhibition. Interestingly, examination of expression data revealed that knocking down ER α or ER β individually resulted in a significant increase in mRNA levels of the opposite receptor (Fig. 4B). Whether an increase in ER β expression compensates for loss of ER α , or vice versa, in mediating raloxifene-induced growth inhibition, or whether raloxifene effects on RT4 cell growth can be mediated by either estrogen receptor cannot be resolved at the present time.

Antiestrogen-induced growth inhibition could be the result of an increase in cell death and/or an arrest of cell proliferation [17]. To test whether apoptosis contributed to the antiestrogen response in RT4 cells, a cell death ELISA was used to measure cytoplasmic nucleosomes following raloxifene administration. Within 24 hours of treatment, DNA fragmentation increased over 6-fold, indicative of an early apoptotic event (Fig. 5A). Use of Z-VAD-FMK, a pan caspase inhibitor, prevented raloxifene-induced cell death, suggesting that this SERM stimulates apoptosis in bladder cancer cells via a caspase-dependent pathway. Subsequent Western blot analysis confirmed caspase-3 cleavage following 6 hours of raloxifene administration (Fig. 5B). PARP cleavage, a downstream indicator of caspase activity, also was induced within 6 hours of ligand treatment (Fig. 5C). Moreover, raloxifene-stimulated PARP cleavage in RT4 cells was blocked by siRNA-mediated depletion of ER α and ER β (Fig. 5D). Together, these data indicate that raloxifene-induced growth inhibition of RT4 cells reflects, at least in part, an ER-dependent increase in apoptosis.

The effect of raloxifene on cell number over time was also evaluated. Consistent with apoptosis induction, observations of RT4 cell growth over a 5-day time course demonstrate that raloxifene reduces viable cells by 40% following 24 hours of ligand exposure (Fig. 6A). However, remaining cells proliferate, and within 48 hours cell numbers recover to nearly 90% of the pre-treatment value. From 48-120 hours, the rate of cell growth was reduced for raloxifene- versus vehicle-treated cultures, suggesting that antiestrogen treatment negatively impacted cell cycle progression. This effect was further assessed by 3H-thymidine incorporation, which indicated a 60% reduction in DNA replication in the presence of raloxifene (Fig. 6B). In contrast to antiestrogen, treatment with the ER agonist E2 had no effect on DNA replication or 5-day RT4 growth (Fig. 6A,B), neither stimulating nor inhibiting cell proliferation.

The cyclin-dependent kinase inhibitor p27 is a negative regulator of cell cycle progression and a key mediator of the anti-proliferation response in tamoxifen-treated MCF-7 cells [34]. Since SERMs can also induce growth inhibition of bladder cancer cells, we evaluated p27 expression in the RT4 line following raloxifene treatment. Western blots revealed an ~2-fold increase in p27 protein 24h after ligand addition (Fig. 6C). Importantly, these changes were not accompanied by an increase in p27 mRNA (Fig. 6D), suggesting the possibility that SERM treatment improved p27 protein stability. Consequently, expression of S-phase

kinase-associated protein 2 (SKP2), whose ubiquitin ligase activity is required for p27 degradation [35], was examined. Results indicated a 50% downregulation of SKP2 mRNA in RT4 cells upon raloxifene administration. These observations suggest that raloxifene increases p27 protein expression by reducing the levels of SKP2, thereby enhancing p27 protein stability. Interestingly, SKP2 mRNA was also decreased in E2-treated RT4 cells (Fig 6D). However, this reduction was not sufficient to impact p27 protein expression (Fig 6C) and is therefore consistent with data from the 5-day time course and ³H-thymidine incorporation assay which demonstrated that E2 had no effect on RT4 cell growth (Fig. 6A,B).

Lastly, the effects of raloxifene on the cell cycle regulators cyclin D1 and BTG2 were examined. Normally upregulated during cell proliferation, cyclin D1 promotes the G1 to S phase transition of the cell cycle. Conversely, expression of the tumor suppressor BTG2 induces growth arrest by negatively regulating cyclin D1 transcription [36]. Following raloxifene treatment, BTG2 mRNA increased by 2-fold in RT4 cells while cyclin D1 expression was reduced by 50% (Fig. 6E). These results, coupled with the p27 and SKP2 data above indicate raloxifene's ability to prevent cell proliferation by altering the expression of cell cycle regulatory genes. Accordingly, raloxifene-induced growth inhibition of RT4 cells represents a combination of both increased apoptosis and decreased cell cycle progression.

Discussion

Antiestrogens have long been used to treat ER α -positive breast cancers, and their use is currently being investigated in the treatment of other ER-positive malignancies including cancers of the lung, colon and prostate, which, unlike the breast, predominantly express ER β [37]. The urinary bladder is also an ER β -positive organ, and expression of this receptor persists in the majority of bladder cancers [11-13], thus making ER β a promising target for novel UCC therapies. Previous reports indicated that the invasive UCC cell line TSU-Pr1 was growth inhibited by raloxifene in a dose-dependent manner, while *in vitro* studies using 5637 cells showed reduced growth in the presence of raloxifene or tamoxifen [24,38]. In this paper we explored the extent to which these antiestrogens could inhibit the growth of multiple UCC cell lines, representing not just invasive tumors but also cancers of non-muscle invasive and metastatic origin. Furthermore, as raloxifene and tamoxifen are both SERMs, the growth response of cells treated with the pure antiestrogen ICI was also assessed. With the exception of the metastatic TCCSUP line, all cell lines tested were growth inhibited at least 20% by two or more antiestrogens. These results suggest that antiestrogen-induced growth inhibition of UCC is widespread and affects cells with a range of tumor stage and grade classifications.

While the data herein highlight the negative impact antiestrogens have on UCC growth, they fail to identify a reciprocal, pro-proliferative estrogenic response. Not only was E2 unable to induce RT4 proliferation, but it was unable to alter expression of the cell cycle genes cyclin D1 and BTG2, two direct targets of ER α transcriptional activity identified in breast cancer [27,39]. Furthermore, although E2 treatment led to a modest reduction in SKP2 expression, this effect was insufficient to impact p27 protein levels. Together these data indicate that the classical E2-ER signaling axis common to breast and other ER α -positive cell types is not active in ER β -positive bladder cells. A similar effect has been described in ER β -expressing colon cancer cells, which are not growth stimulated by E2 treatment but are nonetheless growth inhibited by antiestrogens [40]. A recent report suggested that E2 could induce proliferation of the UCC cell line T24, but the authors did not examine changes in cell number over time, instead inferring growth from short term changes in DNA replication [41]. Moreover, we found that the simultaneous loss of ER α and ER β did not affect the

basal growth rate of RT4 cells, indicating that proliferation of this UCC line occurs independent of ER signaling.

Interestingly, epidemiological reports have identified an apparent association between decreased bladder cancer risk in women and parity, older age at menopause, and increased total years of ovulation [42-44]—factors which reflect greater lifetime estrogen exposure. Additionally, UCC is disproportionately diagnosed in men by a 3:1 ratio [1], indicating that if anything, estrogens may be protective in the development of this disease. However, given that we saw no evidence of this protection in RT4 cells by way of reduced proliferation or increased apoptosis following E2 exposure, the beneficial effect of estrogens on UCC may be limited to pre-neoplastic tissue. Alternatively, estrogens may exert a protective effect in the bladder via an indirect mechanism. Furthermore, while combine estrogen/progesterone hormone replacement therapy has also been variably linked with reduced UCC risk in women [45], neither increased nor decreased incidence of bladder cancer has been reported in women using tamoxifen or raloxifene as a chemopreventive agent for breast cancer [46-48]. However, the incidence of urinary cancer in these populations is very low, and meaningful conclusions regarding SERMs and bladder carcinogenesis are not possible.

Herein initial UCC growth experiments were performed using the MTS assay, a technique that infers proliferation from mitochondrial activity. Results produced by this method may be misleading for compounds that stimulate metabolism but inhibit cell growth. This discordance was previously demonstrated in ICI-treated MCF-7 cells, which exhibit increased mitochondrial respiration despite an arrest of cell cycle progression [29]. According to MTS data, RT4 and TSU-Pr1 cells were growth inhibited by tamoxifen and raloxifene but not by ICI. However, cell counts indicated that much like SERMs, ICI could also inhibit the growth of these cell lines. Additionally, in RT4 cells 4HT and raloxifene both showed a stronger inhibition by cell count compared to MTS assay. Although these experiments examined cell growth at different time points—day 6 vs. 8 for MTS and cell count, respectively—the extent of the disparity is larger than would be anticipated based on cell doubling rates. Consequently, the antiestrogen responses reported here by MTS may underestimate the true growth inhibition produced by these compounds and raise the possibility that antiestrogens impact mitochondrial metabolism in bladder cancer. While the role of ERs in any such mitochondrial dysfunction is unknown, both ER α and ER β have been detected in the mitochondria of certain cell types, including breast and lung cancers [49-51].

Antiestrogens can inhibit cell growth through both ER-dependent and ER-independent mechanisms [16,17]. Accordingly, ligand-responsive cell lines were screened for the presence of ER α and ER β . While only two of seven cell lines were ER α -positive, ER β transcript was detected in all cell lines tested, thus mimicking ER expression profiles seen in primary UCC samples [11-13]. This analysis indicates that ER α expression is not required for the antiestrogen-induced growth inhibition of all UCC cell lines and suggests that ER β may be the principal driver of the antiestrogen response in bladder cancer. Nevertheless, using raloxifene-treated RT4 cells which express both ER α and ER β , we determined that although antiestrogen-induced growth inhibition is ER-dependent, either receptor can mediate this response. Hence, patients with UCC tumors expressing ER of either type may be candidates for antiestrogen treatment.

One way that antiestrogens inhibit cell growth is through the induction of apoptosis. There are two major apoptotic pathways—the extrinsic and the intrinsic pathway—both of which can be triggered by antiestrogen treatment [16,17]. Although these pathways have different initiating stimuli, they converge at the point of effector caspase activation, specifically the cleavage of caspase-3 [52,53]. In the present study using RT4 cells, raloxifene stimulated

the cleavage not only of caspase-3 but also its downstream effector PARP. Moreover, PARP cleavage was reduced in raloxifene-treated cells lacking ER α and ER β , indicating that antiestrogen-induced apoptosis is ER-dependent. Previously, 5637 cells were shown to be growth inhibited by antiestrogen treatment, but the effect of these compounds on cell death was not examined [12]. In contrast, Kim et al. demonstrated that raloxifene treatment of TSU-Pr1 cells led to BAD cleavage and mitochondrial cytochrome c release, but the authors did not test the ER-dependency of this response [38]. Thus, to the best of our knowledge this is the first report of antiestrogen-induced, ER- dependent apoptosis of a bladder cancer cell line.

In addition to stimulating an early apoptotic event, raloxifene also inhibited cell proliferation and altered the expression of cell cycle regulators, including BTG2 and cyclin D1. Identified as targets of ER transcriptional activity in MCF-7 cells [27,39], little is known about the regulation of these genes in cells predominantly expressing ER β . However, in environments overexpressing ER β E2-bound receptor can inhibit cyclin D1 expression while unliganded ER β has been shown to increase expression of BTG2 [54,55], actions consistent with this receptor's anti-proliferative behavior. Conversely, in primarily ER α - positive cells E2 treatment mediates the downregulation of BTG2 while driving cell cycle progression with cyclin D1 induction [27,56]. Antiestrogens can inhibit expression of the latter gene, but BTG2 transcription is unaffected by tamoxifen or ICI [27,57]. In RT4 cells, raloxifene treatment downregulated cyclin D1 expression yet increased BTG2 transcription by 2-fold. These data coupled with reports from the literature support the cell type-specific regulation of ER target genes, an effect that might be related to levels of ER β expression and which may or may not be impacted by ligand treatment.

The role of ERs in bladder cancer is currently unknown. However, in other malignancies primarily expressing ER β , a number of ligand-independent functions for this receptor have been described. For example, in lung cancer unliganded ER β can bind the proapoptotic protein Bad, preventing its interactions with Bcl-2 and inhibiting apoptosis [50]. Herein we demonstrated that antiestrogens induce cell death in RT4 cells in an ER-dependent manner, indicating that a possible function of ER β in bladder cancer is to protect cells from apoptosis. Moreover, as E2 had no effect on the proliferation of this cell line, ER β activity could be ligand-independent similar to that seen in lung cancer cells. Alternatively, antiestrogens may not be inhibiting unliganded ER β but instead inducing a strictly pharmacological response. This alternate hypothesis is supported by evidence indicating that depletion of ER α and ER β does not impact RT4 growth. Nevertheless, irrespective of the normal function of ERs in bladder cells, we believe that UCC represents an additional ER-positive cancer that can be targeted with antiestrogens.

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Fig. 1.

Effect of antiestrogens on the growth of bladder cancer cell lines. Cells were treated with vehicle (Veh; 0.1% ethanol), 1 μ M 4HT, 1 μ M Ral or 1 μ M ICI. Values are shown relative to those of the respective vehicle treatment. A) Proliferation of 5637, RT4, T24, TCCSUP and TSU-PR1 cells determined by MTS assay following six days of antiestrogen treatment. Bars represent the average $\pm$ SEM of at least four experiments. B) Proliferation of RT4 and TSU-PR1 cells assessed by Coulter counter following eight days of antiestrogen treatment. Bars represent the average $\pm$ SEM of three experiments. * $p < 0.05$ compared to vehicle in same cell line.

Fig. 2.

Expression of ER α and ER β in bladder cancer cell lines. Cells were cultured in media containing 10% sFBS for six days and RNA isolated for assessment of (A) ER α or (C) ER β mRNA levels by RT- qPCR relative to the levels ER α and ER β mRNA expressed in MCF-7 and PC-3 cells, respectively. Western blot analysis of (B) ER α or (D) ER β protein expression in whole cell extracts of RT4 cells (15 μ g) as well as MCF-7 (3 μ g) and PC-3 (15 μ g) control cell lines. Actin blots were used as loading controls.

Fig. 3.

Raloxifene-induced growth inhibition of RT4 cells is ER-dependent. Cells were transiently transfected with non-targeting siRNA (siCtrl) or siRNAs targeting both ERα and ERβ, and subsequently treated with vehicle (0.1% ethanol) or 1μM Ral for five days. A) Cell count following ligand treatment. Values are shown relative to those of vehicle-treated cells for each siRNA condition. Bars represent the average ± SEM of four experiments. * $p < 0.05$ compared to the respective vehicle. B) Western blot analysis of ERα and ERβ expression in vehicle-treated siRNA transfected cells. Blot is representative of four experiments. C) Expression of ER mRNA transcripts in vehicle-treated siRNA transfected cells determined by RT-qPCR. Values are shown relative to siCtrl cells with bars representing the average ± SEM of parallel experiments as in (A). # $p < 0.05$ compared to siCtrl cells.

Fig. 4. Raloxifene-induced growth inhibition of RT4 cells can be mediated by either ERα or ERβ. Cells were transiently transfected with non-targeting siRNA (siCtrl) or siRNAs targeting either ERα or ERβ, and subsequently treated with vehicle (0.1% ethanol) or 1μM Ral for five days. A) Number of RT4 cells determined by Coulter counter following ligand treatment. Values are shown relative to those of vehicle- treated cells for each siRNA condition. Bars represent the average $\pm$ SEM of three experiments. * $p < 0.05$ compared to the respective vehicle. B) Expression of ERα (left) and ERβ (right) mRNA in vehicle-treated siCtrl cells determined by RT-qPCR. Values are shown relative to siCtrl. Bars represent the average $\pm$ SEM of parallel experiments as in (A). # $p < 0.05$ compared to siCtrl cells.

Fig. 5.

Raloxifene induces apoptosis in RT4 cells. A) Cell Death ELISA to detect DNA fragmentation in cells treated with vehicle (V; 0.1% ethanol), 1 μ M raloxifene (R) and/or 10 μ M Z-VAD-FMK caspase inhibitor (Z) for 24 hours. Absorbance values are shown relative to those obtained for vehicle-treated cells. Bars represent the average $\pm$ SEM of three experiments. * $p < 0.05$ compared to vehicle. B) Western blot analysis of caspase-3 cleavage or (C) PARP cleavage in RT4 cells treated for 0, 3, or 6 hours with vehicle or 1 μ M raloxifene. D) PARP cleavage in RT4 cells transfected with non-targeting siRNA (siCtrl) or siRNAs targeting ER α and ER β 4 days prior to treating cells for 6 hours with vehicle or 1 μ M raloxifene. Levels of PARP cleavage were assessed by Western blot analysis. All blots are representative of at least three experiments. Tubulin is shown as a loading control.

Fig. 6.

Raloxifene inhibits proliferation of RT4 cells and alters expression of cell cycle regulators. Cells were serum starved for 24h, then treated with vehicle (0.1% EtOH), 1μM Ral or 10nM E2. A) Proliferation time course of RT4 cells as determined by crystal violet incorporation. Cells were treated with ligand for 0, 24, 48, and 120 hours. Values are shown relative to those at time zero, and represent the average $\pm$ SEM of three experiments. B) ³H-thymidine incorporation assay for RT4 cells treated with ligand for 24 hours. Data have been normalized to cell number as determined by crystal violet and are shown relative to vehicle treatment. Bars represent the average $\pm$ SEM of four experiments. C) Representative western blot analysis of p27 expression in cells treated with ligands for 24 hours (*top*). Quantitation analysis of p27 expression (*bottom*). Bars represent the average $\pm$ SEM of three experiments. D) p27 and SKP2, mRNA expression in RT4 cells treated with ligand for 4 hours. Data are shown relative to vehicle-treated cells. Bars represent the average $\pm$ SEM of at least three experiments. E) Same as in (D) except for cyclin D1 and BTG2. * $p < 0.05$ compared to vehicle.