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Abbreviations: DCC: dextran-coated charcoal-treated FCS; ER: estrogen receptor; FCS: fetal calf serum; MTT: thiazolyl blue tetrazolium bromide; TSA: trichostatin A.
ABSTRACT

Most ovarian cancers are estrogen-positive and hormonal treatments using anti-estrogens or aromatase inhibitors are under investigation for treating the tumors that are resistant to conventional therapies. In this study, the long-term effects of two anti-estrogens, namely 4-hydroxytamoxifen and fulvestrant (or ICI182,780), were investigated in ERα-positive BG1 epithelial ovarian cancer cells. To this aim, cells were grown in the presence of anti-estrogen concentrations that were sufficient to saturate the estrogen receptors, but were neither cytotoxic nor cytostatic as indicated by the absence of inhibition of cell proliferation. In these conditions and despite the lack of cytostatic effect of the drugs, long-term treatment (3 months) with the pure anti-estrogen fulvestrant induced a specific, reproducible and irreversible inhibition of ERα expression. This inhibition was accompanied by loss of estrogen-induced cell proliferation and gene expression as indicated by the analysis of several estrogen-responsive genes. ERα down-regulation was not linked to deregulated expression of transcription factors which drive ERα transcription and did not involve DNA methylation or histone deacetylation. Altogether, these results demonstrate that non-cytotoxic concentrations of pure anti-estrogens affect estrogen signaling and might be relevant for the treatment for ovarian cancers.
1. INTRODUCTION

In Western countries, ovarian cancer is the gynecological cancer with the highest morbidity rate. Indeed, more than 22000 new cases are expected to occur in 2012 (United States) and about 15000 women will die of this disease despite a recent increase of the five-year overall survival rate [1, 2]. Besides the development of novel therapies [3], surgery and chemotherapy are currently used as first-line treatments [4, 5]; however, metastases frequently appear and disseminate throughout the peritoneal cavity [6]. A retrospective review concerning 1895 patients with stage III epithelial ovarian cancer reported that the median overall survival was 45.3 months [7]. However, as this value drops to 13-35 months after the first relapse [8], it is important to further refine alternative salvage therapies, especially for relapsed disease.

We and others have shown experimentally that, similarly to breast cancer, some ovarian cancers are hormone-sensitive [9-13]. Indeed, estrogen receptor alpha (ERα) is expressed in more than 60% of primary ovarian cancers [14] and estrogen receptor beta (ERβ) in some of them [15]. Anti-estrogens, such as tamoxifen, inhibit estrogen-induced in vitro growth of ER-positive (ER+) ovarian cancer cell lines [9]. Various hormonal therapies using either anti-estrogens (tamoxifen, fulvestrant) or aromatase inhibitors (letrozole or anastrozole) had shown therapeutic effects in a subset of patients with ovarian cancer refractory to cytotoxic therapies [16-20]. Very few of these studies concerned fulvestrant, although it confers clinical benefits at 90 days to 35% of patients with multiply-recurrent ER+ ovarian cancer, confirming the usefulness of this drug [21, 22]. A more complete and synthetic table detailing all the used endocrine treatments and doses until 2010 is available in a recent review [22]. However, only 5-18% of patients with an ER+ ovarian cancer are sensitive to hormone therapies [23, 24], while 50-60% of ER+ breast cancers respond to such treatments. Ovarian cancers might thus
have an innate resistance to anti-estrogen therapy that could be partially due to the lack of cytostatic effect of these drugs on the growth of ovarian cells. In ER+ breast cancers, anti-estrogen therapy initially blocks the growth of cancer cells, but in 60% of patients such tumors become resistant after few months to several years of treatment [25]. We and others have generated several anti-estrogen resistant breast cancer cell lines by long-term selection in the presence of fixed or progressive doses of anti-estrogens [26, 27]. By contrast, higher doses of anti-estrogens were required to inhibit growth of ovarian cancer cells, such as the BG1 cell line, and very few ovarian cancer models are available for the study of anti-estrogen long-term effects [28].

We were thus interested to study the effects on ERα+ human ovarian epithelial cells (BG1 cell line) of long-term treatment with anti-estrogens (4-hydroxytamoxifen and fulvestrant) at non-cytotoxic and non-cytostatic concentrations (10nM), but sufficient to maintain a full occupancy of the estrogen receptors [26, 42] due to the high affinity of these drugs for such receptors [43, 44]. In such context, cell proliferation is not inhibited by the anti-estrogen compounds and thus phenotypic remodeling is not the result of cell selection based on their cytostatic or cytotoxic effect. Long-term treatment of BG1 cells with fulvestrant permanently inhibited ERα mRNA and protein expression and this effect was accompanied by loss of estrogen-induced cell proliferation and gene expression.
2. MATERIAL AND METHODS

2.1. Chemicals and materials
Culture medium and fetal calf serum (FCS) were obtained from Life Technologies Inc. (Cergy-Pontoise, France). Luciferin (sodium salt) was purchased from Promega (Charbonnières, France). TRIzol reagent and Superscript II reverse transcriptase were from Invitrogen (Cergy Pontoise, France). 17β-estradiol (E2) and Thiazolyl Blue Tetrazolium bromide (MTT) were from Sigma-Aldrich Inc. (St Louis, MO, USA). 4-hydroxytamoxifen (OHTam) and ICI182,780 (7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol or ICI) were a kind gift from Zeneca (Macclesfield, UK). The polyclonal antibodies against ERα (HC20) and Actin were respectively from Santa Cruz Biotechnology (California, USA) and Sigma Aldrich (St Quentin, France). The monoclonal antibody against ERα (1D5) was from Dako (Trappes, France). The anti-rabbit secondary antibody was from Jackson ImmunoResearch (Suffolk, UK) and the Alexa-conjugated secondary antibody was from Life Technologies (Saint Aubin, France). The ECL detection system was from Thermo Fisher Scientific (Illkirch, France) and the Hoechst reagent from Sigma Aldrich (St Quentin, France). For quantitative PCR, SYBR Green Master mix and AB7300 Real-Time PCR system were from Applied Biosystem (Villebon-sur-Yvette, France). For luminescence analysis, the Microbeta Wallac luminometer was from PerkinElmer (Courtaboeuf, France).

2.2. Cell lines and culture conditions
BG1 cells are human ovarian epithelial cancer cells derived from a solid primary tumor from a patient with stage III, poorly differentiated ovarian adenocarcinoma [29] and were obtained from Dr Langdon (Hospital of Edinburgh, S.P. Edinburgh, United Kingdom). The BG1L cell line was originally designed for in vivo experiments in nude mice and was generated by stable
transfection of BG1 cells with a plasmid bearing the luciferase coding sequence under the control of the constitutive CMV promoter. BG1L cells were routinely maintained in DMEM-F12 medium supplemented with 10% FCS (FCS medium). During the selection process, cells were grown in DMEM-F12 (phenol red-free) supplemented with 10% dextran-coated charcoal-treated (DCC) FCS (DCC medium) and with 10nM of the appropriate (OHTam or ICI) anti-estrogen. Before western blotting or mRNA analysis, cells were hormone-deprived in DCC medium for 5 days. The BG1-ERE-Luc cell line (BELZ) was generated by stable co-transfection of BG1 cells with the estrogen-responsive reporter plasmid ERE3-TATA-Luciferase (a gift of Ingemar Pongratz, Karolinska Institute, Huddinge, Sweden) and the pCDNA3.1-zeocin plasmid (Invitrogen, Courtaboeuf, France).

2.3. Transactivation experiments

BELZ cells were seeded at a density of 5 x 10^4 cells per well in 96-well, white opaque tissue culture plates with 150µl culture medium. Hormones were added at the indicated concentration 8hrs after seeding and cells were incubated at 37°C for 16hrs. At the end of incubation, the medium was removed and replaced by test culture medium containing 0.3mM luciferin. Luminescence was measured in intact living cells using a Microbeta Wallac luminometer for 2s.

2.4. Western blot analysis

Whole-cell protein lysates were prepared in WCE buffer (100mM NaCl, 50mM Tris pH 7.4, 5mM CaCl₂, 5mM MgCl₂, 1% NP40, 1% Triton X100). For ERα detection, 40µg of proteins were resolved by 10% SDS-PAGE and then transferred to nitrocellulose membranes using refrigerated Tris-glycine transfer buffer at 30V, overnight. Membranes were incubated with the polyclonal anti-ERα antibody (HC20; 1:4000) for 2hrs and then with the anti-rabbit secondary antibody (1:5000) for 1hr (using 5% fat dry milk in Tris-buffered saline-Tween 20 as blocking agent). In all blots, Actin expression (as loading control) was detected in parallel
with an anti-Actin antibody (1:2000). Detection was carried out with the ECL detection system according to the manufacturer’s instructions. Autoradiographs were scanned, and bands quantified using the Image J software.

2.5. Cell growth analysis

Cells were seeded at a density of about 1000 cells/well in 96-well tissue culture plates in DCC medium. The next day (day 0), cell viability was preliminarily tested by using the MTT assay (n=6 wells), and then 10nM E2, 10nM OHTam, 10nM ICI or vehicle alone (ethanol) were added to the cultures (n= x wells/each condition). Medium was changed every two days. At the indicated time points, 0.5mg/ml MTT was added to selected wells at 37°C for 4hrs. MTT solution was then removed and the plate was air-dried. Formazan crystals were then solubilized in DMSO. Absorbance was read at 540nm on a spectrophotometer using a reference filter of 620nm and results were normalized to the cell density at day 0.

2.6. RNA extraction and quantitative PCR analysis

Total RNA was extracted from cells using the TRizol reagent. Then, 1µg of total RNA was reverse transcribed using the Superscript II reverse transcriptase. Real-time quantitative Polymerase Chain Reaction (qPCR) was performed on an AB7300 Real-Time PCR system. Each reaction included 7.5µL SYBR Green Master mix, 0.1µL of each primer dilution (50µM), 2µL of RT product (1:10 dilution) and H₂O to 15µL. For each sample, results were normalized to the 28S mRNA levels (reference gene).

For ERα promoter analysis, primers were described in [30]: primers 3-5 amplify a region across the exon 1'/exon1 splice site of a transcript generated by promoter B (primer-3 forward, 5’-CACATGCGAGCACATTCCCTCC-3’; primer-5 reverse, 5’-GGACATAACGACTATATGTGTCCAGCC-3’). Primers 4-5 and 4-6 amplify regions of a transcript generated by promoter A (A.1 and A.2 respectively) (primer-4 forward, 5'-CTCGGGCTGTGCTTTTTCC-3; primer-6 reverse, 5'-ACTTCCCTTGT-
CATTGGTACTGGC-3). Primers 7-8 amplify transcripts driven by all promoters (primer-7 forward, 5'-GGACATAACGACTATATGTGGCCAGCC-3; primer-8 reverse, 5'-GGTTGGCAGCTGATGCTCC-3).

Primers used to assess gene expression were as follow: **Fibulin-1** forward, 5'-CCGGATGGCCACTCATCAGA-3', **Fibulin-1** reverse, 5'-CACCACCATCCTGACACA-3'; **FOXA1** forward, 5'-GAAGATGGAAGGGCATGAAA-3', **FOXA1** reverse, 5'-GCCTGAGTTCATGTTGCTGA-3'; **MEF2C** forward, 5'-GGTGGGAGACTGTAAGCCGAA-3', **MEF2C** reverse, 5'-GATTGGCCATACCGTCCCTACCG-3'; **GATA3** forward, 5'-GATGGGACCTTTGGAGAAGG-3', **GATA3** reverse, 5'-GGGGTCGCCTTTGGAGCAGAGA-3'; **USF1** forward, 5'-CACCCTTATTTCCCAGTGCA-3', **USF1** reverse, 5'-GGGTCGCCTTTGGAGCAGAGA-3'; **RIP140** forward, 5'-AATGTGCACTTGAGCCATGA-3', **RIP140** reverse, 5'-TCTGGACACTGCCTGAGAGA-3'; **pS2** forward, 5'-GGGGTCGCCTTTGGAGAAGG-3', **pS2** reverse, 5'-GGGGTCGCCTTTGGAGAAGG-3'; **Cyclin D1** forward, 5'-GATGGGACCTTTGGAGAAGG-3', **Cyclin D1** reverse, 5'-GGGGTCGCCTTTGGAGAAGG-3'; **Cyclin E** forward, 5'-GATGGGACCTTTGGAGAAGG-3', **Cyclin E** reverse, 5'-GGGGTCGCCTTTGGAGAAGG-3'; **Cyclin A** forward, 5'-GATGGGACCTTTGGAGAAGG-3', **Cyclin A** reverse, 5'-GGGGTCGCCTTTGGAGAAGG-3'; **Cyclin B1** forward, 5'-GATGGGACCTTTGGAGAAGG-3', **Cyclin B1** reverse, 5'-GATGGGACCTTTGGAGAAGG-3'; **Cyclin B2** forward, 5'-GATGGGACCTTTGGAGAAGG-3', **Cyclin B2** reverse, 5'-GATGGGACCTTTGGAGAAGG-3'.

### 2.7. Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde, permeabilized in 1% Triton X-100 and incubated with 1% bovine serum albumin at room temperature for 3h. Cells were then incubated with
the monoclonal anti-ERα antibody (1:100) overnight and the receptor-antibody complex was detected using an Alexa-conjugated secondary antibody diluted at 1:1000. After washing, sections were counterstained with Hoechst and mounted for fluorescence analysis with an AxioPlan2 (Zeiss) microscope. Data were analyzed by using the AxioVision software. Negative controls (rabbit or mouse IgGs alone) were performed and no staining was observed in these conditions. Quantifications of ERα staining in cells are the mean ± S.D. of three fields (20X magnification, with about 100 cells visible in each analyzed field) from three independent experiments.

2.8. Statistics

Data are presented as mean ± standard deviation (SD). For Figures 2C, 3C, 4A-D, data are the mean ± SD of triplicates for BG1L cells, and the mean ± SD of the three different cultures (LT0, LT1 and LT2) for BG1L-ICILT and/or BG1L-ICILT cells.

Statistical analysis included one-way analysis of the variance (ANOVA) followed by the Bonferroni’s post-hoc test in order to determine significant differences concerning cell proliferation experiments (Fig 2E), and image analysis of autoradiographs for ERα expression (Fig 1A and 3B). Statistically significant differences of the relative fluorescence levels (Fig 3D) were tested using the Mann-Whitney test. Differences in cell proliferation of Fig 1C and 2B as well as differences in gene expression profiles from qPCR experiments were determined using the Student’s t-test. For all tests: * p<0.05, ** p< 0.01.
3. RESULTS

3.1. Estrogen responsiveness of BG1 ovarian cancer cells

By western blot analysis, the estrogen-responsive BG1 ovarian epithelial cancer cells showed significant ERα expression which was clearly regulated by hormone treatment (Fig 1A) as observed in other cell types [31]. Specifically, treatment with the partial anti-estrogen 4-hydroxytamoxifen (OHTam) led to ERα accumulation, whereas 17β-estradiol (E2) and particularly the pure anti-estrogen fulvestrant (ICI182,780 or ICI) down-regulated ERα expression. Using BG1 cells stably transfected with a luciferase gene under the control of an estrogen responsive element (BELZ cells), we confirmed the strong effect of E2 on ER-mediated transactivation. Conversely, ICI had no effect on ER transcriptional activity, whereas OHTam behaved as a strong partial agonist and exhibited 50% of the maximal activity obtained in the presence of 10nM E2 (EC₅₀ of 60nM) (Fig 1B).

The effect of E2 and of the two anti-estrogens on BG1 cell growth was then investigated by using the MTT assay and the bioluminescent BG1L cell line in which luciferase expression is driven by the CMV promoter. BG1L cell growth was clearly enhanced by E2 (Fig 1C). Conversely and in agreement with the result of the ER-mediated transactivation assay, OHTam had partial agonist activity, whereas the pure anti-estrogen ICI had no effect on growth when compared to vehicle alone.

3.2. Long-term treatment of BG1 ovarian cancer cells with anti-estrogens

We then analyzed the effects of long-term culture of BG1L cells in the presence of anti-estrogens at concentrations that are devoid of cytotoxic and cyostatic effects, but sufficient to maintain a full occupancy of the estrogen receptors. To this aim, BG1L cells were divided in
six independent cultures that were cultured in the presence of 10nM of OHTam (BG1L-OHTLT) (n=3) or ICI (BG1L-ICILT) (n=3) for 3 months (Fig 2A).

In the absence of ER ligands, BG1L-OHTLT cells grew significantly faster than parental BG1L cells (Fig 2B). By contrast, the proliferation rate of BG1L-ICILT cells was slightly lower. Analysis of the mRNA expression of different Cyclin genes (Fig 2C) showed that, in BG1L-OHTLT cells, CCND1 and CCNE were expressed at comparable level as in BG1L cells, whereas CCNA and CCNB1 were weakly but significantly down-regulated. In BG1L-ICILT cells, CCND1 mRNA level was significantly reduced, whereas CCNB2 expression was slightly but significantly increased compared to parental cells. As CCND1 has a key role in the release from the G0/G1 block, its reduced expression in BG1L-ICILT cells is in accordance with the lower growth rate of these cells; conversely, such relationship was not observed in BG1L-OHTLT cells. Therefore, these results show no clear evidence for a direct link between Cyclin expression and the differences in growth rates observed upon treatment with the two anti-estrogens.

The growth properties of these cell lines were then compared in response to exposure to ER ligands. This was done in parallel in the three independent cultures of BG1L-OHTLT and BG1L-ICILT cells in order to ensure that any phenotypic change associated with anti-estrogen treatment was reproducible. After 7 days of culture, the three BG1L-OHTLT cell lines displayed a similar response to 24hr hormonal treatment (Fig 2D). More precisely, OHTam had partial agonistic activity, whereas ICI had no effect on cell proliferation as observed in parental BG1L cells (see Fig 1B). Conversely, the three BG1L-ICILT cell lines almost lost their ability to respond to both E2 and OHTam (Fig 2E). Indeed, although a weak, but significant growth effect of E2 and OHTam was observed in two (LT0 and LT2) of the three independent BG1L-ICILT cultures, it was much lower than in BG1L-OHTLT cells,
suggesting that long-term culture in the presence of ICI leads to a phenotypic change that is reproducibly different from the one caused by OHTam.

3.3. Long-term treatment with fulvestrant inhibits ERα expression

We then assessed ERα expression in the different BG1L cell lines obtained after long-term anti-estrogen treatment by western blotting. Compared to parental BG1L cells, ERα level was slightly increased in BG1L-OHTLT cells and strongly reduced in BG1L-ICILT cells (for hormone-deprived cells (Fig 3A), or following exposure to ER ligands (Fig 3B)). Importantly, the significant decrease of ERα expression was comparable in the three independent BG1L-ICILT cultures (Fig 3A and B), indicating that the phenotypic changes induced by the long-term ICI treatment were reproducible.

Quantification of ERα mRNA expression in the different cell lines confirmed the results obtained by western blotting. Indeed, ERα mRNA level was higher in BG1L-OHTLT cells and lower in BG1L-ICILT cells in comparison to parental BG1L cells (Fig 3C).

The decrease of ERα content in BG1L-ICILT cells could be due to a homogenous, modest decrease in all cells, or to a strong decrease (or even a total loss) in a specific sub-population. To answer this question, immunofluorescence analysis was performed by using an anti-ERα antibody (Fig 3D). In accordance with the results obtained by western blotting, quantification of the immunofluorescence data with the Axiovision software confirmed that ERα expression was higher in BG1L-OHTLT and lower in BG1L-ICILT cells in comparison to BG1L cells. Moreover, the reduction of ERα expression in BG1L-ICILT cells was homogeneous and did not correspond to the clonal emergence of an ER-negative sub-population.

In order to better characterize ERα down-regulation in BG1L-ICILT cells, a kinetic analysis of ERα expression was performed by western blotting using BG1L cells that were harvested at different time points after addition of ICI to the DCC culture medium (Fig 3E). ERα
expression decreased progressively during 4 months, but the major reduction was observed already after one month of treatment (approximately 15 cell doublings), thus indicating an early reshaping of these cells upon long-term ICI treatment.

3.4. Expression of estrogen-responsive genes in BG1-derived cell lines

In order to monitor ERα activity in BG1L-OHTLT and BG1L-ICILT cells, the expression of several endogenous estrogen-responsive genes (Trefoil factor 1 also known as pS2, Fibulin-1 and RIP140) was analyzed by quantitative RT-PCR.

In cultures that were hormone-deprived for 5 days, the levels of Fibulin-1 and RIP140 were comparable in all cell lines, whereas pS2 expression was much higher in BG1L-OHTLT cells (Fig 4A). Upon E2 treatment, accumulation of pS2, Fibulin-1 and RIP140 was not significantly different in BG1L and BG1L-OHTLT cells (Fig 4B). By contrast, in the BG1L-ICILT cell line, the effect of E2 on pS2, Fibulin-1 and RIP140 expression was significantly reduced in comparison to what observed in BG1L and BG1L-OHTLT cells. This suggests that ERα activity is no longer regulated by E2 in BG1L-ICILT cells.

3.5. Expression of ERα transcriptional regulators in BG1-derived cell lines

In order to further investigate the mechanisms involved in the reduced expression of ERα by long-term ICI treatment of BG1L cells, we measured by quantitative PCR in hormone-deprived cells, the mRNA levels of several transcription factors (USF1, FOXA1, GATA3, and MEF2C) that are involved in the regulation of the ERα promoter [32, 33] (Fig 4C). Expression of USF1 and GATA3 was comparable in BG1L and BG1L-OHTLT and BG1L-ICILT cells. Conversely, FOXA1 was strongly up-regulated and MEF2C down-regulated in BG1L-OHTLT cells in comparison to BG1L or BG1L-ICILT cells. The expression of these two transcription factors was not significantly different in BG1L-ICILT cells and the parental
BG1L cell line, suggesting that their deregulated expression is probably not the causal event of the decreased expression of ERα upon long-term ICI treatment. Interestingly, addition of E2 negatively regulated (to different extents) USF1, FOXA1, GATA3 and MEF2C mRNA expression in the parental BG1L cell line (Fig 4D). As observed for pS2, Fibulin-1 and RIP140, this E2 negative regulation was lost in the BG1L-ICILT cell lines, thus confirming the impact of long-term ICI treatment on estrogen regulation of gene expression.

3.6. Global regulation of ERα expression upon long term ICI treatment of BG1 cells

The generation of ERα transcripts is a complex process that involves at least seven promoters [34, 35]. Changes in ERα expression levels could be due to global regulation of all promoters, or to the specific inactivation of only few of them. As in breast cancer cell lines, regulation of ERα transcription by E2 mainly involves the A and B proximal promoters [36, 37], we used oligonucleotides that specifically amplify transcripts originating from the A and B promoters of the ERα gene (see Fig 5A and [30]) to determine which promoters are inhibited in BG1L-ICILT cells. The level of ERα transcripts generated by the A and B promoters was strongly decreased in BG1L-ICILT cells in comparison to BG1L cells (Fig 5B). This was also the case when universal oligonucleotides (primer set 7-8) were used, thus confirming the data shown in Fig 3C and suggesting that probably all transcripts were inactivated as a consequence of long-term ICI treatment.

We then investigated whether ERα inhibition in BG1L-ICILT cells was irreversible. Cells were hormone-deprived in DCC medium for 5 days and then their ERα protein level determined and compared to that of hormone-deprived cells that were then switched to FCS medium (the medium used for parental BG1L cells) or to DCC medium with ICI for another 7 days (Fig 5C). ERα expression was comparable in the three conditions, indicating that no
release of inhibition occurred even after 12 days of culture in the absence of ICI (5 days in DCC medium alone and 7 days in FCS medium).

The irreversible ERα inhibition might be the result of chromatin remodeling toward a closed structure induced by DNA and/or histone modifications. However, treatment of BG1L-ICILT cells with 2.5 μM of the DNA methylation inhibitor 5-aza-2'-deoxycytidine for 6 or 9 days had no effect on ERα protein level (Fig 5D). A 22hr supplementary treatment with the histone deacetylase inhibitor TSA resulted in a decrease rather than in an increase of ERα protein level. Such inhibitory effect of TSA on ERα expression, even when used alone, has been observed in various cell types and particularly in BG1 cells (data not shown). Altogether, these data suggest that DNA methylation and/or histone modification are not the main mechanisms involved in the down-regulation of ERα expression in BG1L-ICILT cells.
4. DISCUSSION

Despite the poor response of ovarian cancers to tamoxifen (5-18% of patients), second-line hormonal therapies may have some utility [38] and relatively little toxicity [16, 17, 21]. In this study, we show that non-cytostatic concentrations of the triphenylethylene derivative 4-hydroxytamoxifen and of the pure antagonist fulvestrant have different effects on BG1 ovarian cancer cells, with a specific inhibition of ERα expression after long-term treatment with fulvestrant.

BG1 ovarian cancer cells exhibit a poor response to anti-estrogens. Indeed, OHTam has mitogenic activity at low concentrations (below 100nM) and cytostatic effect on cell proliferation only at concentrations above 1µM [9, 28]. However, such high cytostatic concentrations are difficult to reach in clinical practice and this might explain the poor response of ovarian cancers to tamoxifen-based therapies. As a comparison, the clinical use of tamoxifen in breast cancer patients leads to intra-tumoral concentrations of 4OHTam (the active metabolite of tamoxifen) between 77nM [39] and 180nM [40].

Another consequence of the poor response of BG1 ovarian cancer cells to OHTam is that very high concentrations of OHTam (raising progressively to 16 µM) are needed to select resistant cells on the basis of its cytostatic effect [28]. Despite the doses used, after 5 months of OHTam treatment, ERα levels were not significantly altered in such cells. Similarly, in MCF-7 breast cancer cells, most of the resistant colonies (picked after a 21-day treatment with 1µM OHTam followed by a 7-day drug-free recovery period) had ERα levels that were comparable to those of the parental cell line [41]. In the present work, we assessed the effect of long-term exposure to anti-estrogens on BG1 ovarian cancer cells using low doses (10nM) of such compounds that were however sufficient to occupy the estrogen receptors [26, 42]. In these conditions, we found a slight increase of ERα protein expression after 3 months of treatment
with 10nM OHTam. Together, these results indicate that in breast and ovary cancer cells, neither high nor low doses of 4-hydroxytamoxifen down-regulate ERα expression.

Fulvestrant (ICI182,780), a pure anti-estrogen that is now used as a second line hormone therapy for breast cancer, has different effects on breast, ovaries or uterus [45, 46]. At 100 nM, the proliferation of MCF-7 breast cancer cells is inhibited, whereas BG1 cells or Ishikawa endometrial cells are unaffected [47, 48]. Long-term effects of exposure to low doses of ICI also appear to be different in BG1 and MCF-7 cells. In the MCF-7 cell line, a 21-day treatment with 0.1 μM of ICI was not accompanied by ERα disappearance in the majority of the clones analyzed (similarly to what reported for OHTam) [41]. In our laboratory, clonal MCF-7 cells treated with 10nM ICI for up to 4 months also showed ERα expression levels that were comparable to those of untreated cells (data not shown). Conversely, in BG1 cells, ICI treatment led to a significant decrease of ERα expression (Fig 3), suggesting a clear difference between breast and ovarian cells in their response to ICI treatment.

It should be stressed that the effect of ICI on ERα expression is specific because, in BG1L cells that were cultured in DCC medium alone for a long time, ERα expression and regulation were identical to those of BG1L parental cells (data not shown). Moreover, the loss of ERα expression observed in BG1L cells upon long-term exposure to ICI was reproducible since it was observed in three independent cell cultures, suggesting that this effect was not the result of the selection of cells which underwent a random mutational process, but rather of a progressive reshaping of genetic expression. This hypothesis is strengthened by the fact that ERα expression driven by the most active of the ERα promoters was altered in these cells, thus suggesting that a long range modification of the chromatin structure might be concerned. Moreover, the clonal origin of the parental BG1L cell line (which was selected just after insertion of the luciferase gene) possibly led to a more homogenous cell population than in the initial BG1 cell line from which they were derived. The long-term ICI effect on ERα
expression in three independent cultures of BG1L cells is thus more likely to be due to a
global phenotypic reshaping, rather than the result of a pure selection process which in
addition would have been hampered by the lack of cytostatic effect of the drug. Interestingly,
the regulation induced by a 24hr treatment with ER ligands was still observed, but was less
important than in the parental cell line or in cells treated with OHTam (Fig 3B). This is in
accordance with the post-translational nature of this hormonal regulation [31], which occurs
only on the residual receptors of these cells.

As expected, the decrease in ERα expression was accompanied by loss of estrogen regulation
on cell proliferation and on the expression of several estrogen responsive genes, such as pS2,
Fibulin-1 and RIP140. Moreover, the mRNA levels of several transcriptional regulators of the
ERα promoter (USF1, FOXA1, GATA3 and MEF2C), which were down-regulated by E2 in
BG1L parental cells, were also no longer modulated by E2 in cells upon long-term treatment
with ICI. The “pioneer factor” FOXA1, which is involved in the translation of epigenetic
marks, also controls, in conjunction with the transcriptional regulator GATA3, a gene network
that is particularly active in the luminal subtype A breast cancer [49]. FOXA1 is also a better
predictor of survival for breast cancer patients than the progesterone receptor, however no
significant differences in breast cancer-specific survival were observed in patients with
cancers characterized by high or low FOXA1 expression and treated with tamoxifen [49, 50].
Interestingly, FOXA1 was up-regulated in BG1L-OHTLT cells and, although to a lesser
extent, also in BG1L-ICILT cells, suggesting that it might act as an adapter piece in the
cellular reshaping induced by long-term anti-estrogen treatment of ovary cancers.

Regulation of ERα expression is a complex process that involves at least seven different
promoters, alternative splicing and complex post-translational modifications
(phosphorylation, sumoylation, acetylation, polyubiquitination) [35]. Multiple mechanisms
involved in ERα silencing in many tumors have been identified. In a significant fraction of
breast cancers, loss of $ER_\alpha$ expression was found to be the result of CpG island hypermethylation within the $ER_\alpha$ promoter [51]. In BG1L-ICILT cells, we did not find evidences that DNA methylation was involved in the reduction of $ER_\alpha$ expression upon long-term ICI treatment (Fig 5D), indicating that other mechanisms might play a role in this reshaping phenomenon.

In precedent studies, we reported the irreversible inactivation of an estrogen-responsive luciferase reporter gene by long-term OHTam treatment of MCF-7 breast cancer cells [52, 53]. No CpG methylation of the transgene was observed during this inactivation process; conversely, Heterochromatin Protein 1 (HP1) was associated with the inactivated form of the transgene [54]. Changes in miRNA regulation could also be involved in the effect of ICI on $ER_\alpha$ expression [55]. Two putative miR-206 sites were found in silico within the 3'-untranslated region of human $ER_\alpha$ mRNA [56]. However, miR-206 expression level was slightly lower in BG1L-ICILT cells than in BG1L cells, suggesting that this miRNA is probably not responsible for $ER_\alpha$ down-regulation in such cells (data not shown). Nevertheless, it would be interesting to analyze other miRNAs, such as miR-221/222 which have been reported to confer fulvestrant-resistance in breast cancer cells [57].

In addition to their biological significance, these findings might have potential clinical implications. Indeed, our data reveal that long-term treatment of BG1 cells with non-cytostatic concentrations of fulvestrant is accompanied by a significant and reproducible decrease of $ER_\alpha$ levels and estrogen response in term of cell proliferation and target gene expression. In addition to its effect on estrogen signaling, long-term treatment with ICI also reduced cell proliferation (Fig 2B) in comparison to untreated cells (BG1L) or cells treated with tamoxifen (BG1L-OHTLT). In conclusion, these data suggest that fulvestrant at low doses might be more beneficial than tamoxifen for hormonal treatment of patients with
ovarian cancers. However, further studies are required to validate these observations in the clinical context.

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FIGURE LEGENDS

Figure 1: Estrogen responsiveness of BG1 cells.

A) ERα expression was assessed by western blotting in BG1 cells treated with EtOH (vehicle), 10nM E2, OHTam or ICI for 24hrs. Quantification was performed as described in Materials and Methods. Data (mean ± SD of three independent experiments) are expressed as the percentage of the value in control BG1 cells (vehicle). B) BG1 cells stably transfected with an ERE-luciferase reporter construct (BELZ cells) were treated with E2, OHT and ICI at the indicated concentrations for 24hrs. Induction of ERE-mediated luciferase activity is expressed relative to the maximal E2 response set at 100%. C) Cell proliferation was assessed in BG1-Luc (BG1L) cells treated with 10nM ER ligands or vehicle alone (control) as described in Materials and Methods (mean ± SD of sixplicates).

Figure 2: Growth properties and expression of Cyclin genes in BG1 cells after long-term treatment with anti-estrogens.

A) BG1L cells were divided into six independent cultures. Three of them were continuously treated with 10nM OHT and the other three with 10nM ICI in DCC medium for 3 months. The resulting independent cultures were named BG1L-OHTLT and BG1L-ICILT (LT=long-term treated). B) Analysis of cell proliferation in parental BG1L, BG1L-OHTLT and BG1L-ICILT cells after hormone (mean ± SD of sixplicates). C) Quantitative PCR analysis using total RNA from parental BG1L, BG1L-OHTLT and BG1L-ICILT cells in hormone-deprived conditions (error bars significance in the Statistics section). D and E) Cell proliferation was measured in the three independent cultures (LT0, LT1 and LT2) of BG1L-OHTLT (panel D) or BG1L-ICILT cells (panel E) grown in the presence of the indicated ER ligands (mean ± SD of sixplicates).
Figure 3: Decreased expression of ERα after long-term treatment with 10nM ICI182,780.

A) ERα expression was assessed by western blotting in hormone-deprived BG1L, BG1L-OHTLT and BG1L-ICILT cells. B) The different cell lines were treated with the indicated ER ligands or vehicle alone for 24hrs before analysis of ERα expression as described in Materials and Methods. Data are expressed as the percentage of the value in control BG1L-OHTLT cells (vehicle) and are the mean ± SD of the percent values from LT0, LT1 and LT2 cultures. C) Cells were treated as in B before being processed for qPCR analysis of ERα mRNA expression as described in Materials and Methods (error bars significance in the Statistics section). The values for untreated cells (Control) were set at 1 for all cell lines. D) Immunofluorescence analysis of ERα expression was performed as described in Materials and Methods in hormone-deprived BG1L, BG1L-OHTLT and BG1L-ICILT. The histogram corresponding to the fluorescence quantification is presented in the lower panel (mean ± SD of n=10). E) ERα expression was assessed by western blotting in BG1L cells treated with 10nM ICI in DCC medium for the indicated period of time.

Figure 4: Expression of estrogen-responsive genes and of ERα transcriptional regulators in BG1, BG1L-OHTLT and BG1L-ICILT cells.

A) BG1L, BG1L-OHTLT and BG1L-ICILT cells were hormone-deprived and placed in DCC medium containing vehicle alone (EtOH) for 24hrs and then the expression of estrogen-responsive genes (pS2, Fibulin-1 and RIP140) was assessed by qPCR analysis as described in Materials and Methods. B) Same analysis as in A) after treatment or not with E2 for 24hrs. C) Same analysis as in A) for the expression of ERα transcriptional regulators (USF1, FOXA1, GATA3 and MEF2C). D) Same analysis as in A) after treatment or not of BG1L and BG1L-
ICILT cells with E2 during 24hrs. The values for untreated cells (Control) were set at 1 for all cell lines. See the error bars significance of the whole figure in the Statistics section.

**Figure 5: Analysis of ERα down-regulation.**

A) Schematic representation of the two proximal promoters of the ERα gene, as described in [30]. Broken lines show the splicing events and arrows indicate the position of the different oligonucleotides used for the specific amplification of transcripts originating from the A and B promoters. B) BG1L, BG1L-OHTLT and BG1L-ICILT cells were hormone-deprived for 5 days and then processed for qPCR analysis using the different primer sets shown in A (error bars significance in the Statistics section). C) ERα expression was analyzed by western blotting in BG1L-ICILT cells that were hormone-deprived for 5 days (lane 1), and then cultured in FCS medium with phenol red (Rol) (lane 2) or in DCC medium with 10nM ICI (lane 3) for another 7 days. D) BG1L-ICITLT cells were hormone-deprived for 6 days (lane 1, 2) or 9 days (lane 3, 4). Cells were then treated with 2.5μM 5-azacytidine (Aza) for 3 days (lane 2) or with 2.5 μM 5-azacytidine for 3 days and then with 100ng/ml TSA for 22hrs (lane 4).
REFERENCES

Figure 1

A

A Western blot analysis of ERα expression in Ctrl, E2, OHT, and ICI treated cells. ERα and Actin bands are shown.

B

A graph showing the concentration-response relationship for BELZ transactivation. The x-axis represents the concentration (log M) and the y-axis represents the % of transactivation (% max E2). The bars indicate the statistical significance of the differences between treatments.

C

A graph showing cell proliferation in BG1L cells treated with Control, E2, OHT, and ICI. The x-axis represents the number of days and the y-axis represents cell proliferation (arbitrary units). The plots show the increase in cell proliferation over time for each treatment.
Figure 2

A

BG1L

ICI 182,780

3 months

BG1L-ICILT (T0, T1, T2)

OHTam

BG1L-OHTLT (T0, T1, T2)

B

Cell proliferation (arbitrary units)

Days

BG1-L

BG1-L-OHTLT

BG1-L-ICILT

0 3 5 7

C

Relative mRNA level

Cyclin

D1 E A B1 B2

BG1L

BG1L-OHTLT

BG1L-ICILT
D

![Graph showing cell proliferation for BG1L-OHTLT](image)

- **Control**
- E2
- OHT
- ICI

Cell proliferation (% control)

T0 | T1 | T2
---|---|---

E

![Graph showing cell proliferation for BG1L-ICILT](image)

- **Control**
- E2
- OHT
- ICI

Cell proliferation (% control)

T0 | T1 | T2
---|---|---

**,**
C

![Graph showing relative ERα mRNA level](image)

D

![Images showing ERα and DAPI staining](image)

![Graph showing relative fluorescent level](image)
Days of treatment

BG1L

ERα

Actin

0  7  30  90  120
Figure 4

A

B

Relative mRNA level

pS2  |  Fibulin-1  |  RIP40

BG1L  |  BG1L-OHTLT  |  BG1L-ICILT

E2

Control  |  BG1L  |  BG1L-OHTLT  |  BG1L-ICILT

Relative mRNA level

pS2  |  Fibulin-1  |  RIP40

**  |  **  |  **

*  |  *  |  NS

NS  |  NS  |  **

NS  |  **  |  **
C

Relative mRNA level

BG1L
BG1L-OHTLT
BG1L-ICILT

USF1 FOXA1 GATA3 MEF2C

D

BG1L

BG1L-ICILT

Ctrl E2

Relative mRNA level

USF1 FOXA1 GATA3 MEF2C