Molecular changes associated with the agonist activity of hydroxy-tamoxifen and the hyper-response to estradiol in hydroxy-tamoxifen-resistant breast cancer cell lines

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Abstract

The aim of this study was to explore the pharmacological response to 4-hydroxy-tamoxifen (OH-Tam) and to estradiol (E_2) in three cell lines: MVLN, a human breast carcinoma cell line derived from MCF-7, and two MVLN-derived OH-Tam-resistant (OTR) cell lines, called CL6.8 and CL6.32. The OH-Tam response in the OTR cells was associated with the development of both an agonist activity of the drug on cell proliferation and the resistance of the cells to OH-Tam-induced apoptosis. The OTR cells also developed an increased sensitivity to the E_2 growth-stimulating activity. To delineate the genes that determine such responses, we combined a mini-array-based gene-selection approach and an extensive real-time quantitative PCR exploration in the MVLN and OTR cell lines exposed to three pharmacological conditions: a 4-day treatment with E_2 , OH-Tam or both E_2 and OH-Tam. Compiled data revealed a hyper-response to E_2 and a modification of the OH-Tam pharmacological response (loss of antagonist action and agonist activity) at the gene-expression level. The proteins encoded by the genes selected in this study have been reported to be involved in the regulation of cell proliferation, cell transformation, DNA repair and apoptosis, or belong to the ErbB/epidermal growth factor receptor-driven pathway. Our data also provide evidence of changes in transcriptional co-regulator expression, elevated mitogen-activated protein kinase activity and increase in the phosphorylation status of estrogen receptor α on serine residue 118 in the OTR cell lines, suggesting the possible involvement of such mechanisms in the agonist activity of OH-Tam and/or the hyper-response of cells to E_2 . Taken together, our study should enhance our knowledge of the multifactorial events associated with the development of Tam resistance in two independent cell lines issued from the same selection process and should help in the identification of potential molecular targets for diagnosis or therapy.

Endocrine-Related Cancer (2005) 12 75–92

Introduction

The estrogen receptor α (ER α) plays a crucial role in the clinical care of breast cancer patients, and measurement of the level of ER α expression has allowed an accurate prediction of the response to endocrine therapy. The selective estrogen receptor modulator

(SERM) tamoxifen (Tam) and its active metabolite 4-hydroxy-tamoxifen (OH-Tam) have been shown to be an effective adjuvant therapy for ER α -positive tumors by reducing the incidence of contralateral breast cancer by 50% (EBCTC Group 1992). However, despite its widespread use in clinical practice, acquired resistance develops in almost all Tam-treated

women after long-term medication (Johnston 1997). The molecular mechanism leading to Tam resistance is not fully understood, and multifactorial changes leading to a survival system for the cancer cells seem to be involved, rather than a gain- or loss-of-function mechanism (Brockdorff *et al.* 2003). Cellular disturbances have been reported as being possibly involved in the emergence of Tam resistance, such as modifications in Tam metabolism (Osborne 1993), ER mutations (Mahfoudi *et al.* 1995), altered ER α or ER β expression (Johnston *et al.* 1995, Speirs *et al.* 1999), qualitative and/or quantitative changes in transcriptional co-repressors or co-activators (Girault *et al.* 2003a, Osborne *et al.* 2003) and phosphorylation of ER α by mitogen-activated protein kinase (MAPK; Kato *et al.* 1995), phosphoinositide 3-kinase/Akt (Campbell *et al.* 2001) or protein kinase A (Le Goff *et al.* 1994, Michalides *et al.* 2004). There is also compelling evidence that inappropriate activation of growth factor signaling cascades (e.g. by over-expression of heregulins, transforming growth factor α (TGF α), epidermal growth factor receptor (EGFR) and HER2/erbB2 (Liu *et al.* 1995, Lupu *et al.* 1996, Nicholson *et al.* 2001)) could promote endocrine resistance. Supporting data show that MAPK activity is increased in breast cancer cell-line models of endocrine resistance (Coutts & Murphy 1998) and correlates with a shorter duration of response to endocrine therapy in clinical breast cancer (Gee *et al.* 2001). During the progression of Tam resistance, an important step is the loss of the anti-estrogen activity of Tam and the passage to an agonist activity of Tam-ER complexes (Gottardis & Jordan 1988, Ring & Dowsett 2004). Possible mechanisms involved in the emergence of such agonist activity of the drug are modification of the ER α conformation or activity by phosphorylation of key regulatory sites in the AF-1 domain (Ser-118) or the hinge region (Ser-305) (Kato *et al.* 1995, Michalides *et al.* 2004) and qualitative and/or quantitative changes in transcriptional co-regulators (Shang & Brown 2002).

Today, information emerging from data *in vitro* and *in vivo* provides evidence that under deprivation of estrogens or blockade of estrogen action with Tam, breast cancer cells also adapt by changing their response to estradiol (E₂) and by developing an increased sensitivity to its growth-stimulating action (Martin *et al.* 2003, Bernstein *et al.* 2004, Santen *et al.* 2004). This so-called hypersensitivity to E₂ was shown to be associated with increased concentrations of ER α and ER-mediated events (Santen *et al.* 2001, Chan *et al.* 2002). The presence of elevated levels of MAPK or Akt activity was also shown to be involved in the

development of E₂ hypersensitivity (Yue *et al.* 2002, 2003). Interestingly, long-term exposure of MCF-7 cell xenografts to Tam enhances sensitivity to both E₂ and the estrogenic effects of Tam (Bernstein *et al.* 2004), suggesting that these two events are potentially linked.

MVLN is an ER α -positive and hormone-responsive human breast carcinoma cell line derived from MCF-7 (Demirpence *et al.* 1993). Treatment of MVLN cells for 6 months allowed the emergence of OH-Tam-resistant (OTR), but still estrogen-dependent, individual cellular clones that have not been fully characterized (Badia *et al.* 2000). Large-scale studies of gene expression using cDNA or oligonucleotide arrays have been performed successfully to select molecular targets associated with estrogen regulation (Vendrell *et al.* 2004) or with antagonist and agonist activity of SERMs (Hodges *et al.* 2003, Frasor *et al.* 2004). In this study we aimed to identify variations in gene expression associated with the OH-Tam and E₂ pharmacological responses of two MVLN-derived OTR cell lines because such variations might play a critical role in the development or the signature of OH-Tam resistance. Two OTR cell lines, namely CL6.8 and CL6.32, were explored concomitantly, with the purpose of delineating the common and different molecular events acquired by two individual cellular clones issued from the same selection process. By combining mini-array gene selection and extensive real-time quantitative PCR (RTQ-PCR) exploration, we identified specific signaling pathways and genes associated with an agonist activity of OH-Tam and/or a hyper-response to E₂ in the two OTR cell lines studied. As modifications of MAPK activity, modification of the phosphorylation status of ER α and quantitative changes in transcriptional co-regulators could be mechanisms contributing to the emergence of such responses, we explored these mechanisms in the two OTR cell lines. This study provides for the first time an extensive exploration of the multifactorial events associated with the development of OH-Tam resistance in a resistant cell model and should enhance our knowledge of the cancer cell-regulatory pathways involved in both the agonist activity of OH-Tam and the hyper-response to E₂.

Materials and methods

Cell culture

MVLN, CL6.8 and CL6.32 cells were grown during one or two passages as described previously (Demirpence *et al.* 1993), then purged for 4 days in Dulbecco's

modified Eagle's medium without Phenol Red and supplemented with 3% steroid-depleted, dextran-coated, charcoal-treated fetal calf serum (DCC medium). The cells were then treated for 4 days (with one media change) under the pharmacological conditions described below.

Cell-cycle distribution analysis by flow cytometry

The cells were purged in DCC medium for 4 days and then grown for 4 days in the presence of vehicle, 1 nM E₂, 200 nM OH-Tam or both 1 nM E₂ and 200 nM OH-Tam. The cells were harvested and washed twice with PBS. 10⁶ cells were then incubated with 1 ml DNA staining solution (25 µg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100) for 24 h at 4 °C. Propidium iodide fluorescence of 20 000 nuclei was analyzed for each sample by FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). The percentage of cells within the G₀/G₁, S and G₂/M phases of the cell cycle were identified by analysis with the Modfit LT™ software (Verity Software, Topsham, ME, USA).

Cell-proliferation analysis

The cells were purged in DCC medium for 4 days and then plated at 12 000 cells/well on a 96-well tissue-culture plate in DCC medium. 1 day later the cells were treated for 1, 3, 5 or 8 days with vehicle, 1 nM E₂ or 200 nM OH-Tam. Proliferating cells were analyzed using the Cell Proliferation ELISA, 5-bromodeoxyuridine (BrdU; colorimetric) Kit (Roche, Meylan, France). Briefly, the cells were labeled for 24 h with BrdU and the labeled nuclei were identified using a specific anti-BrdU antibody according to the manufacturer's recommendations.

Detection of apoptosis by annexin V staining

The cells were purged in DCC medium for 4 days and then grown for 4 days in either the absence of any treatment or the presence of 600 nM of OH-Tam. Apoptotic cells were detected using the Annexin-V-FLUOS Staining Kit (Roche). Briefly, cells were harvested, washed twice with PBS and 10⁶ cells subsequently labeled with fluorescein isothiocyanate (FITC)-annexin V according to the manufacturer's recommendations. FITC fluorescence of 20 000 cells was then analyzed by flow cytometry. The percentage of apoptotic cells was determined by analysis with CellQuest™ software (Becton Dickinson).

Neutral Red cytotoxicity assay

The cells were plated at 12 000 cells/well on a 96-well tissue culture plate in DMEM medium and treated for 4 days with different concentrations of OH-Tam (10⁻⁹ to 10⁻⁴ M). After 4 days of treatment the medium was removed and the cells were incubated for 3 h with Neutral Red at 33 mg/l. Cells were then washed twice with PBS and lysed in the presence of 1% acetic acid/50% ethanol. After 15 min of gentle shaking the absorbance was measured at 540 nM. The uptake of Neutral Red is proportional to the number of viable cells.

Hybridization of complex probe and cDNA-array data analysis

Variations in gene-expression levels were analyzed by large-scale measurement with home-made nylon cDNA mini-arrays (7.5 × 11.5 cm; 1019 human genes; 12 genes/cm²) produced in our facility (TAGC Laboratory, CIML, University of Aix-Marseille II, Marseille, France) as described previously (Bertucci *et al.* 1999, Vendrell *et al.* 2004). Spotted targets were single amplified PCR products amplified from control clones and IMAGE cDNA clones (IMAGE Consortium, Cambridge, UK). The human cDNA clones were selected on the basis of practical criteria (Bertucci *et al.* 1999), and the genes were chosen because of a proven or putative implication in cancer (oncogenes and tumor suppressors, apoptosis and cell-cycle regulators, transcription factors and transcriptional co-regulators, DNA-repair regulators, cytokines, growth factors, adhesion molecules, nuclear receptors, enzymes, etc.) The list is available at <http://tagc.univ-mrs.fr/pub/Cancer/>. Total RNA was extracted by CsCl ultracentrifugation and RNA integrity was checked by denaturing agarose-gel electrophoresis. Hybridization to each array with a ³³P-labeled probe synthesized by reverse-transcribing 5 µg total RNA was performed as described previously (Bertucci *et al.* 1999). Array data scanning, quantification and normalization were performed as described previously (Vendrell *et al.* 2004); hybridization signals were scanned with a FUJI BAS 5000 beta imager (Raytest, Asnieres, France), then quantified with the HDG Analyzer software (Genomic Solution, Ann Arbor, MI, USA) by integrating all spot pixel intensities and removing a spot background value determined in the neighboring area; intensity values were adjusted by a normalization step based on the DNA quantification of each spot and the sum of intensities detected in each experiment. Expression values were expressed as the ratio of the

treated cell line gene value to that obtained with the corresponding untreated cell line (the ratio was called fold change or FC).

RTQ-PCR analysis

RTQ-PCR measurements were performed as described previously (Girault *et al.* 2003a, Vendrell *et al.* 2004) using a LightCycler® (Roche) or an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) using the corresponding SYBR Green Kit, according to the manufacturer's recommendations. For each set of primers, a standard curve was made with serial dilutions from a control cDNA sample, and high RTQ-PCR efficiency rates were verified for each investigated transcript (>90%). We also checked that our RTQ-PCR measurements were reproducible (the inter-assay variation was less than 5%, as calculated by the s.e.; data not shown). All measurements were normalized to the expression of either the 28 S ribosomal gene (Vendrell *et al.* 2004) or the TATA box-binding protein (*TBP*) gene (Girault *et al.* 2003a).

Western blotting

Cells were lysed in the presence of 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM dithiothreitol, 52 mM NaF, 40 mM glycerol 2-phosphate disodium salt hydrate, 0.1 mM sodium orthovanadate and protease inhibitor in 20 mM Tris, pH 7.5, 150 mM NaCl, 10 mM Hepes and 5 mM EDTA. For each sample, 50 µg total protein was separated on SDS/PAGE gels before transferring to a PVDF membrane (Sigma-Aldrich, St Quentin Fallavier, France). Membranes were then incubated with the appropriate primary antibody, and detection was performed as recommended by the manufacturer with horseradish peroxidase-conjugated secondary antibody using the ECL Plus™ western blotting detection reagents (Amersham Biosciences, Orsay, France). The ERα antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the bcl2 antibody from Lab Vision Corporation (Fremont, CA, USA); cyclin A2 and tubulin antibodies were from Sigma Chemical Co. (Saint-Louis, MO, USA); the Bak antibody was from Calbiochem (Darmstadt, Germany); the PCNA antibody was from eBioscience (San Diego, SA, USA) and the phospho-p44/42 MAPK, p44/42 MAPK, survivin and phospho-ser118 ERα antibodies were from Cell Signaling (Beverly, MA, USA).

Results

Relative resistance to OH-Tam and functionality of ER-mediated transcription in CL6.8 and CL6.32 cell lines

OH-Tam dose-response experiments (Neutral Red cytotoxicity assay) were performed to measure the concentration giving rise to a 50% reduction in cellular viability (IC₅₀ values). The IC₅₀ values (means ± s.d.) from three independent experiments were 1.5 ± 0.28, 8.5 ± 1.3 and 8.6 ± 0.93 µM in the MVLN, CL6.8 and CL6.32 cell lines, respectively. These data demonstrate that the CL6.8 and CL6.32 cell lines both displayed a 5.7-fold relative resistance to OH-Tam compared with MVLN (Student's *t*-test; *P* < 0.0001). One of the possible mechanisms that could lead to OH-Tam resistance is altered expression of ERα and ERβ (Johnston *et al.* 1995, Speirs *et al.* 1999). In accordance with previous observations (Badia *et al.* 2000), we confirmed that ERα protein expression was lower (approximately 6-fold) in CL6.8 and CL6.32 cells than in MVLN cells (data not shown). Investigation of the ERβ mRNA level by RTQ-PCR demonstrated an expression level that was 1000-fold lower than that of ERα mRNA and no difference in expression between the three cell lines (data not shown). We also validated the functionality of the ERα and the basal transcription machinery in MVLN, CL6.8 and CL6.32 by verifying that E₂ treatment elicited an increase in Trefoil factor 1 (TFF1) (pS2) expression in the three cell lines at both the mRNA and the protein levels, and that OH-Tam was able to counteract this effect (data not shown).

E₂ exerts a hyper-response and OH-Tam an agonist activity on the proliferation of the OTR CL6.8 and CL6.32 cells

To investigate the effect of E₂ and OH-Tam exposure on cell proliferation of MVLN, CL6.8 and CL6.32 cells we first assessed cell-cycle distribution by flow-cytometry analysis. Figure 1 reveals that treatment with E₂ stimulated a great percentage of cells to enter to S phase in the three cell lines (Fig. 1, compare panel A with B, E with F and I with J). However, the proportion of cells in the S phase under E₂ treatment was greater in the two OTR cell lines than in the MVLN cells (35 vs 26%; Fig. 1 compare panel F and J with B). Investigation of the growth kinetics of MVLN, CL6.8 and CL6.32 cells under E₂ exposure (Fig. 2A) also highlighted differences between the MVLN and the OTR cells: (i) the E₂-stimulated cell growth was significantly detectable on day 5 for the

Figure 1 Cell-cycle distribution of MVLN, CL6.8 and CL6.32 cells in response to E₂ and OH-Tam. Cells were treated over a period of 4 days with vehicle, 1 nM E₂, 200 nM OH-Tam or both E₂ (1 nM) and OH-Tam (200 nM) as described in the Materials and methods section. Cells were then stained with propidium iodide, and nuclei were analyzed for DNA content by flow cytometry. Values shown are from one experiment that was reproduced at least three times. The percentage of cells in the G0/G1, S and G2/M phases are indicated on the pie charts.

MVLN cells ($P < 0.01$) but earlier (day 3) for the CL6.8 and CL6.32 cells ($P < 0.05$); (ii) the amplitude of E₂-stimulation of cell proliferation was significantly greater in the two OTR cell lines than in the MVLN cells (days 3, 5 and 8).

We next investigated the proliferating response of MVLN, CL6.8 and CL6.32 cells under OH-Tam exposure. OH-Tam treatment of MVLN cells was able to totally antagonize the E₂-stimulatory effect on S-phase entry and to induce growth arrest in G₀/G₁ (Fig. 1C and D). On the contrary, OH-Tam exposure of the CL6.8 and CL6.32 cells was associated with a higher proportion of cells in the S phase than in MVLN cells (27 versus 10%, Fig. 1, compare panel G and K with C). Exposure of the two OTR cell lines under both E₂ and OH-Tam treatments gave patterns of cell distribution identical to those obtained under OH-Tam alone (Fig. 1, compare panels H and L with G and K respectively), suggesting the absence of any additive or synergic effect between E₂ and OH-Tam in the two OTR cells. The growth kinetics assay illustrated in Fig. 2B confirmed that, in agreement with a previous study (Semlali *et al.* 2004), OH-Tam was able to stimulate the proliferation of the two OTR cell lines.

Taken together, our findings demonstrate an increase of amplitude of E₂ stimulation of cell proliferation (hyper-reponse to E₂) and estrogenic-like effects of OH-Tam (agonist activity) in the OTR CL6.8 and CL6.32 cell lines.

Global assessment of the E₂ and OH-Tam responses in the OTR CL6.8 and CL6.32 cell lines by cDNA-array analysis

With the aim of selecting specific genes or signaling pathways associated with the agonist activity of OH-Tam and the hyper-response to E₂, we performed global gene expression profiling using a 1019 human gene mini-array on MVLN and OTR CL6.8 and CL6.32 cells exposed for 4 days to four different pharmacological conditions: no treatment, E₂, OH-Tam or treatment with both E₂ and OH-Tam. We demonstrated previously that selection of gene-expression variations based on a cut-off value (called fold change or FC) of 1.7 combined with three independent cell-culture replicates gave reliable results that could be validated at both the mRNA and protein levels (Vendrell *et al.* 2004). We thus identified genes whose expression was E₂-regulated ($FC \geq 1.7$) in MVLN, CL6.8 and CL6.32 cell lines; 50 upregulated genes and 26 downregulated genes resulted from this selection (results not shown).

We then assessed the agonist activity of OH-Tam on these 76 genes by identifying the proportion of genes responding to the following criteria: $FC \geq 1.7$ under OH-Tam exposure and $FC \geq 1.7$ under the pharmacological conditions combining both E₂ and OH-Tam exposure. We then defined, as proposed by Frasor and colleagues (Frasor *et al.* 2004), full agonistic activity

Figure 2 Growth kinetics of MVLN, CL6.8 and CL6.32 cells. MVLN (white bars), CL6.8 (black bars) and CL6.32 (grey bars) cells were grown in DCC medium, then treated for 1, 3, 5 or 8 days with vehicle, 1 nM E₂ (A) or 200 nM OH-Tam (B). The proliferative response was assessed by BrdU labeling. Results are expressed as a percentage of cells incorporating BrdU in the presence of treatment compared with untreated cells. Results are means $\pm$ s.d. from three independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001 versus the corresponding MVLN treatment according to Student's t -test.

(if OH-Tam alone evoked >70% of E₂ activity and the reversion of E₂ activity by OH-Tam was <30%) and partial agonist action (if OH-Tam alone evoked activity >35% but $\leq$ 70% of E₂ activity, and if the effect of E₂ could be reversed by OH-Tam by 30–50%). Antagonist action of OH-Tam was defined by an FC value of <1.7 under OH-Tam treatment and an FC value of <1.7 under both E₂ and OH-Tam treatment. The data presented in Fig. 3 revealed that OH-Tam elicited an antagonist action in the MVLN cells on the majority of the E₂-regulated genes as well as a subsequent agonistic action (16 and 23%, considering the up- and downregulated genes respectively), in accordance with previous observations (Hodges *et al.* 2003, Frasor *et al.* 2004). The OH-Tam response of the two OTR cell lines was strikingly different. Indeed, both CL6.8 and CL6.32 cells demonstrated an increase

in the global OH-Tam agonistic action on the E₂-upregulated genes (38 and 30%, respectively, compared with the 16% detected in MVLN cells), mainly due to an increase in the full agonist activity of the drug. Considering the E₂ down-regulated genes, a slight increase in the OH-Tam full agonist action was noted in the two OTR clones compared with the MVLN cells (19.2 vs 11.5%), whereas the global agonistic activity of OH-Tam did not show any difference.

We then assessed variations in the amplitude of the estrogenic response for the 76 E₂-regulated genes by considering arbitrarily that this latter was increased in CL6.8 and CL6.32 cells when an E₂-induced variation of gene expression was superior or equivalent to 200% of the corresponding variation measured in E₂-treated MVLN cells. An increase in the amplitude of the

Figure 3 OH-Tam response of genes up- ($n=50$) or down-regulated ($n=26$) by E₂. OH-Tam activity was defined as: full agonism (black segments) if the FC value was ≥ 1.7 under OH-Tam treatment, if FC ≥ 1.7 under both E₂ and OH-Tam treatment, if OH-Tam alone evoked $>70\%$ of E₂ activity and if reversion of E₂ activity by OH-Tam was $<30\%$; partial agonism (grey segments) if FC ≥ 1.7 under OH-Tam treatment, if FC ≥ 1.7 under both E₂ and OH-Tam treatment, if OH-Tam alone evoked activity $>35\%$ but $\leq 70\%$ of E₂ activity, and if reversion of the E₂ effect by OH-Tam was $30\text{--}50\%$; antagonism (white segments) if FC <1.7 under OH-Tam treatment and FC <1.7 under both E₂ and OH-Tam treatment.

estrogenic response was identified in the OTR cells for a substantial proportion of the genes: 40% of the upregulated genes and 23% of the downregulated genes in CL6.8 cells, and 30% of the upregulated genes and 19% of the downregulated genes in CL6.32 cells. The amplitude of the estrogenic response detected in these genes ranged from 200 to 862%. Moreover, we also identified several other genes whose expression varied under E₂ treatment in the OTR cells but not in the MVLN cells (2.5% of the genes investigated in our study).

In conclusion, global assessment by cDNA array of gene-expression variations detected under E₂ and OH-Tam treatment suggested an increased agonist activity of OH-Tam and an increased response to E₂ in both OTR cell lines. A great proportion of the genes associated with these two events were genes coding for proteins involved in cell proliferation, cell-cycle regulation, cell transformation and apoptosis. We thus decided to further explore by RTQ-PCR the signaling pathways identified by the array data. Figure 4 illustrates validation by RTQ-PCR of particular gene-expression variations selected by cDNA-array analysis and demonstrates an excellent consistency between the cDNA-array results and the RTQ-PCR

measurements (as we observed previously (Vendrell *et al.* 2004)).

E₂ exerts a hyper-response at the level of gene expression in the OTR CL6.8 and CL6.32 cell lines

Table 1 illustrates compiled RTQ-PCR data measuring the expression variations of 36 selected genes in the MVLN, CL6.8 and CL6.32 cell lines under E₂, OH-Tam or both E₂ and OH-Tam treatment. Analysis of the data revealed at the gene-expression level a hyper-response to E₂ in the OTR cell lines, defined by (i) the detection of E₂-induced gene-expression variations that were not measured in MVLN cells (*GADD45A*, *STK6/Aurora-A*, *CDKN1A/p21/Cip1*, *EGR2/KROX20*, *MBD2*, *DNMT3B*, *TRADD*, *TRAF1*, *TNFSF10/APO2L/TRAIL*) or (ii) the increase of the amplitude of the E₂ response measured in MVLN cells (ranging from 200 to 1370% of the E₂ response detected in MVLN cells). Taken together, 61 and 39%, respectively, of the gene-expression variations measured in E₂-treated CL6.8 and CL6.32 cell lines displayed at the gene-expression level a hyper-reponse to E₂, and 86% of the hyper-response to E₂ detected in CL6.32 cells

Figure 4 Comparison of the gene-expression variations (FC) measured by cDNA arrays (black bars) and RTQ-PCR (white bars) after 4 days of E₂, OH-Tam or both E₂ and OH-Tam treatment of MVLN, CL6.8 and CL6.32 cells. The RTQ-PCR values indicated are means from at least three independent experiments.

was also present in CL6.8 cells. Gene-expression variations associated with the hyper-reponse to E₂ affected upregulated genes encoding proteins associated with stimulatory effects on cell proliferation or transformation (e.g. *PCNA*, *STK6/Aurora-A*, *CCNA2*, *CCNB2*, *CCNE2*, *MYBL2*, *PTTG1/Securin*, *MAD2L1*, *TERT*) or with negative effects on apoptosis (*BCL2*, *BIRC5/Survivin*), and downregulated genes encoding proteins known to exert suppressive effects on growth (*GADD45A*, *CDKN1A/p21/Cip1*, *EGR2/KROX20*, *TGFB2*) or pro-apoptotic action (*EGR2/KROX20*, *TGFB2*, *TRADD*, *TRAF1*, *TNFSF10/APO2L/TRAIL*, *BAK*). Hyper-response to E₂ was validated by Western-blot analysis in CL6.8 cells for cyclin A2, PCNA, Bak, survivin and Bcl2 proteins and represented 810, 190, 190, 550 and 220%, respectively, of the E₂ response detected in MVLN cells (data not shown). The correlation between expression variations

detected at the mRNA level and at the protein level validated the relevance of the expression modulations we identified under E₂ exposure.

Gene-expression variations associated with OH-Tam activity in MVLN cell line

We further decided to focus on the OH-Tam effects on the expression of genes presented in Table 1. In the MVLN cell line, antagonistic action of OH-Tam was the main phenomenon, as OH-Tam was able to counteract 89% of the E₂-induced modulations of gene expression. In previous studies, a 'reverse pharmacology' (defined as genes that were specifically regulated by OH-Tam but either unaffected by E₂ exposure or inversely regulated by E₂) has been identified for only a few genes (Montano & Katzenellenbogen 1997, Hodges *et al.* 2003, Frasor *et al.* 2004). Our data newly

Table 1 Gene-expression variations associated with the hyper-response to E₂ and the OH-Tam agonist activity in the OTR CL6.32 and CL6.8 cells. Gene-expression variations were assessed on MVLN, CL6.32 and CL6.8 cells grown for 4 days in the presence of E₂ (1 nM), OH-Tam (200 nM) or both E₂ (1 nM) and OH-Tam (200 nM). The expression values presented are expressed in terms of fold change (FC) values (calculated by dividing the treated cell line gene-expression value by that obtained for the corresponding untreated cell line) and represent means from at least two independent RTQ-PCR measurements. Gene-expression variation was considered to be significant for FC ≥ 2 or FC ≤ -2. Upregulations are indicated in bold, downregulations are underlined and hyper-responses to E₂ detected in CL6.32 or CL6.8 cells are indicated in italics.

FC value											
Gene name	Symbol	Accession no.	MVLN		CL6.32				CL 6.8		
			E ₂	OH- Tam	E ₂ +OH- Tam	E ₂	OH- Tam	E ₂ +OH- Tam	E ₂	OH- Tam	E ₂ +OH- Tam
Antigen identified by monoclonal antibody Ki-67 Proliferating cell nuclear antigen Growth arrest and DNA-damage-inducible, α Serine/threonine Kinase 6	MI67	NM_002417	2.79	-1.09	-1.06	4.95	3.00	3.58	7.70	4.37	5.88
	PCNA	NM_002592	2.20	-1.73	-1.43	4.46	2.75	2.28	3.01	3.53	3.53
	GADD45A	NM_001924	-1.09	-1.37	1.29	-2.53	-2.03	-1.82	-4.55	-2.01	-1.90
	STK6	NM_003600	1.58	-1.15	-1.58	3.47	2.00	2.60	4.94	2.76	2.39
	CCNA2	NM_001237	3.76	1.10	-1.40	13.02	4.09	2.06	8.20	3.87	3.66
	CCNB2	NM_174264	4.50	-2.13	-2.00	9.09	4.99	4.59	14.33	7.24	3.45
	CCNE2	NM_057749	2.50	-1.50	-1.50	3.02	2.60	3.46	6.34	3.97	4.63
	CCND2	NM_001759	-12.47	-1.51	-1.36	-9.42	-3.14	-2.77	-23.44	-1.69	-1.54
	CCND1	NM_053056	2.53	1.46	1.54	1.55	1.01	1.23	1.72	1.19	1.41
	CDKN1A	NM_000389	-1.62	1.53	1.35	-2.63	-1.53	-1.53	-4.73	-1.64	-1.56
Nephroblastoma-overexpressed gene Topoisomerase (DNA) Iα, 170kDa Hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	NOV	NM_002514	-17.15	-1.35	-1.48	-17.30	-4.24	-3.51	-28.38	-2.35	-1.68
	TOP2A	NM_001067	2.85	-1.33	-1.51	4.93	2.27	2.44	9.13	3.94	4.25
	HPRT1	NM_000194	3.72	-1.04	-1.48	3.88	2.00	1.25	2.22	1.74	1.54
	BRCA2	NM_000059	4.45	-1.22	-1.26	6.04	3.49	2.80	4.71	2.87	4.26
	RAD51	NM_002875	5.17	-1.15	-1.46	4.34	3.01	3.14	5.20	6.46	3.75
	MYBL2	NM_002466	2.39	1.46	1.00	5.72	4.56	6.15	12.47	4.23	11.13
	PTTG1	NM_004219	2.45	1.04	-1.08	8.91	4.54	4.17	8.98	3.13	3.33
	MAD2L1	NM_002358	3.16	1.12	1.33	4.74	3.05	3.43	7.04	3.20	3.90
	TERT	NM_003219	2.33	-1.42	-1.47	21.61	6.32	6.95	31.93	3.88	6.31
	EGR2	NM_000399	-1.58	-1.80	-1.09	-5.25	-5.03	-3.35	-12.11	-3.53	-3.17
Early growth response 2 Zinc-finger protein 217 Methyl-CpG-binding-domain protein 2 DNA methyltransferase 3β Cystatin A Transforming growth factorβ2 Transforming growth factorβ3 Amphiregulin	ZNF217	NM_006526	-3.15	1.35	-1.46	-3.95	-2.81	-5.58	-12.82	1.02	-1.46
	MBD2	NM_003927	-1.84	-1.62	-1.79	-4.52	-2.16	-2.66	-1.76	-1.28	-1.05
	DNMT3B	NM_006892	1.23	-1.68	-1.34	2.50	2.00	2.30	1.90	1.20	1.90
	CSTA	NM_005213	-18.07	-1.50	-4.25	-15.07	-3.94	-2.55	-8.99	-2.36	-2.27
	TGFB2	NM_003238	-14.28	6.16	5.01	-6.25	1.05	1.00	-50.00	1.01	-1.19
	TGFB3	NM_003239	-2.60	1.47	1.05	-1.23	1.20	-1.21	-4.35	-1.31	-1.78
	AREG	NM_001657	97.87	1.18	3.08	52.86	3.71	4.56	41.80	3.16	4.04
	TNFRSF6	NM_000043	4.43	1.50	-1.11	5.75	3.55	2.09	4.07	1.12	1.35
	TRADD	NM_003789	-1.51	2.20	2.14	-2.73	-1.34	-1.45	-4.30	-1.70	-1.30
	TRAF1	NM_005658	-1.03	1.22	1.26	-1.03	-1.73	-1.49	-6.19	-2.64	-3.13
Tumor necrosis factor receptor-associated factor 1 Tumor necrosis factor superfamily, member 10 Bcl2-interacting killer B-cell CLL/lymphoma 2 Bcl2-antagonist/killer 1 Bcl2-binding component 3 Baculoviral IAP repeat-containing 5	TNFSF10	NM_003810	1.13	1.84	2.07	-1.50	1.21	1.60	-24.00	-2.20	-3.01
	BIK	NM_001197	-5.40	2.25	2.12	-5.44	-1.31	-1.83	-5.00	1.03	-1.08
	BCL2	NM_000633	2.00	1.24	1.55	2.85	2.22	2.51	4.00	2.48	2.23
	BAK1	NM_001188	-5.13	-2.49	-2.20	-7.42	-2.39	-2.70	-12.98	-3.53	-2.10
	BBC3	NM_014417	-8.05	1.64	1.32	-3.33	-1.6	-2.56	-7.42	-2.00	-2.77
	BIRC5	NM_001168	2.02	-1.06	-1.35	4.6	2.85	2.74	9.56	3.75	4.43

Figure 5 Western-blot analysis of OH-Tam agonist activity on the expression of PCNA, cyclin A2, survivin and Bcl2 proteins. Protein expression was analyzed as described in the Materials and methods section using specific antibodies in cells untreated (-) or treated for 4{Q4}days with 1 nM E₂ or 200 nM OH-Tam. Tubulin protein level was measured to verify even loading.

identified a reverse pharmacology of OH-Tam for the *CCNB2*, *TGFB2*, *TRADD* and *BIK* genes in the MVLN cell line.

OH-Tam elicited an agonist activity at the gene expression level in the OTR CL6.8 and CL6.32 cell lines

Contrary to what was observed in the MVLN cell line, OH-Tam agonistic activity (i.e. an 'estrogen-like' signature) was the main phenomenon identified in the two OTR cell lines, and represented 83% of the gene expressions presented in Table 1. The OH-Tam signature was very close to that obtained under both E₂ and OH-Tam exposure, suggesting the absence of any additive or synergic effect between E₂ and OH-Tam in the two OTR cells. Of much interest was the function of the genes associated with the agonist activity of OH-Tam in the CL6.8 and CL6.32 cells (Table 1). Indeed, agonist activity of OH-Tam was associated with (i) the upregulation of the expression of genes coding for proteins involved in positive stimulation of cell proliferation or in cell-cycle regulation

(*PCNA*, *STK6/Aurora-A*, *CCNA2*, *CCNB2*, *CCNE2*, *MAD2L1*, *AREG/Amphiregulin*), in DNA replication and genomic stability (*TOP2A*, *HPRT1*, *TERT*), in DNA repair (*BRCA2*, *RAD51*) and in cell transformation (*MYBL2*, *PTTG1/Securin*), (ii) the downregulation of the expression of genes encoding proteins involved in negative regulation of cell proliferation (*GADD45A*, *NOV*, *EGR2/KROX20*). The most striking example was that of the *CCNB2* gene, encoding a protein which regulates G₂/M transition, on which OH-Tam exerted a reverse pharmacology in MVLN cells but agonist activity in the CL6.8 and CL6.32 cells. Validation at the protein level of the OH-Tam agonist activity on cyclin A2 and PCNA proteins is illustrated in Fig. 5.

Interestingly, no agonistic action of OH-Tam was observed on the expression of *CCND1*, at the mRNA (Table 1) or protein (data not shown) level, which codes for a protein known to play an essential role in E₂-induced cell-cycle progression. Furthermore, RTQ-PCR measurements of the mRNA levels of other genes involved in the regulation of cell proliferation (*CCND3*, *CCNE1*, *CDKN1B*, *WEE1*) demonstrated

no variation of expression under any of the pharmacological conditions tested (data not shown). Taken together, these observations suggest that these five genes do not seem to be involved in the development of OH-Tam resistance.

The OTR CL6.8 and CL6.32 cells are not responsive to OH-Tam-induced apoptosis

Tam has been reported to have anti-proliferative effects but also to increase cell death (Zhang *et al.* 1999). We thus decided to explore the impact of OH-Tam treatment on cell death in the two OTR cell lines. Apoptotic cells were stained with FITC–annexin V and analyzed by flow cytometry in three independent experiments. We confirmed that OH-Tam was able to elicit cell death in MVLN cells (the proportion of apoptotic cells was 14.0% in OH-Tam-treated cells compared with 8.9% in untreated cells; $P=0.001$). Interestingly, the gene-expression variations presented in Table 1 revealed that OH-Tam exposure of MVLN cells was associated with the upregulation of three pro-apoptotic genes, *TGFB2*, *TRADD* and *BIK*. On the contrary, OH-Tam treatment did not elicit any significant apoptotic response in the CL6.8 and CL6.32 cell lines. Indeed, the proportion of apoptotic cells was 7.7% in OH-Tam-treated CL6.8 cells compared with 8.0% in untreated CL6.8 cells, and 4.2% in OH-Tam-treated CL6.32 cells vs 4.4% in untreated CL6.32 cells. At the gene-expression level, we observed (Table 1) (i) the absence of OH-Tam upregulation of *TGFB2*, *TRADD* and *BIK* expression, (ii) OH-Tam agonist activity associated with the downregulation of four pro-apoptotic genes coding for two members of the death receptor signaling (*TRAF1*, *TNFSF10/APO2L/TRAIL*), the transcription factor *EGR2/Krox-20* and *PUMA* (encoded by *BBC3/PUMA*, a p53 target gene) and (iii) OH-Tam agonist activity associated with an increased expression of anti-apoptotic genes coding for the Bcl2 protein and the survivin protein (*BIRC5/Survivin* gene). Validation of agonist activity of OH-Tam on Bcl2 and survivin protein expression is illustrated in Fig. 5. The proportion of apoptotic cells detected by flow cytometry was significantly lower ($P=0.0003$) in untreated CL6.32 cells vs (4.4%) than in untreated MVLN cells (8.9%). Interestingly, we found that Bcl2 levels were more elevated in untreated CL6.32 cells than in untreated MVLN cells, both at the mRNA (data not shown) and protein (Fig. 5) levels. These findings suggest that Bcl2 could be one of the major actors in the survival network developed by the OTR cell lines during their acquisition of OH-Tam resistance. Besides the data presented in Table 1, we

also measured the expression variation of other genes involved in the regulation of apoptosis (*FADD*, *CRADD*, *TNFRSF10A*, *TNFRSF10B*, *TNFRSF10C*, *BAX*, *APAF1* and *DAP3* genes), and no significant variation was observed under the pharmacological conditions tested (data not shown).

The OTR cell lines display at the basal level increased MAPK activity, elevated status of phosphorylation of ER α Ser-118 and over-expression of genes members of the ErbB-driven pathway

The importance of the MAPK pathway has been demonstrated in the development of anti-estrogen resistance, in Tam agonist activity and in hypersensitivity to E₂ (Kato *et al.* 1995, Coutts & Murphy 1998, Kurokawa *et al.* 2000, Yue *et al.* 2002). We thus assessed the MAPK activity in MVLN, CL6.8 and CL6.32 cells at the basal level (i.e. in the absence of any treatment; Fig. 6A). MAPK activity (expressed as the ratio of active MAPK to total MAPK) was higher in CL6.8 and CL6.32 cells than in MVLN cells (respectively 216 and 171% of the MAPK activity detected in MVLN cells). The MAPK pathway is able to target and phosphorylate key regulatory sites on the ER protein, notably within the ligand-independent trans-activation AF-1 domain, and Ser-118 is a putative target site for phosphorylation by MAPK (Bunone *et al.* 1996). The data presented in Fig. 6B demonstrate that elevated MAPK activity in the two OTR cell lines was also accompanied by an increase in the basal phosphorylation of ER on Ser-118 (expressed as the ratio of phospho-ER α to total ER α).

Previous studies have demonstrated that over-expression and/or activation of growth factor ligands and growth factor receptors could promote endocrine resistance and activate the MAPK pathway (Kurokawa *et al.* 2000, Nicholson *et al.* 2001). We therefore decided to explore gene-expression mRNA levels of the four members of the ErbB family (*erbB1/EGFR*, *erbB2/HER2/neu*, *erbB3/HER3*, *erbB4*) and specific ErbB-specific ligands (*EGF*, *AREG/Amphiregulin*, *NRG1*, *NRG2*, *NRG3*, *NRG4*, *BTC*, *DTR/HBEGF*, *EREG*, *TGFA*, *Epigen*). Of great interest was the identification in CL6.8 and CL6.32 cells of the over-expression of *EGFR* and numerous *ErbB*-specific ligands. Indeed, RTQ-PCR measurements of MVLN, CL6.8 and CL6.32 cells revealed an increase in the basal expression of *EGFR* (FC, +3.1), *AREG/Amphiregulin* (+2.8), *BTC* (+2.0) and *DTR/HBEGF* (+2.9) genes in CL6.8 cells and an increase of the basal expression of *Epigen* in both CL6.8 and CL6.32 cells (FC, +12.5 and +25.0,

Figure 6 Increase of MAPK activity (A) and the phosphorylation status of the ER α Ser-118 (B) in both OTR cell lines in comparison with MVLN cells. MVLN, CL6.8 and CL6.32 cells were grown for 4 days in DCC medium. Protein levels of phosphorylated MAPK, total MAPK, phosphorylated ER α Ser-118 and total ER α were immuno-detected by Western blot. The levels of activated MAPK and the phosphorylation status of the ER α Ser-118 were determined by densitometry scanning. The values are means from two independent experiments.

respectively). Furthermore, OH-Tam was demonstrated to exert an agonist effect leading to the over-expression of the *AREG/Amphiregulin* gene in the CL6.8 and CL6.32 cell lines (Table 1), reinforcing the basal over-expression of this gene in both cell lines. No gene-expression variation was detected for the *erbB2/HER2/neu*, *erbB3/HER3*, *erbB4*, *NRG2*, *EREG* or *TGFA* genes, and *NRG1*, *NRG3* and *NRG4* gene expression was undetectable in the three cell lines studied (data not shown).

Expression and regulation of ER α co-regulators in MVLN, CL6.8 and CL6.32 cell lines

Several studies have suggested that changes in expression levels of cofactors might contribute to enhanced E₂-stimulated transcription and OH-Tam agonist activity (Smith *et al.* 1997, Shang & Brown 2002). Furthermore, E₂ is able to modulate the expression of some co-factors, suggesting that the existence of such regulatory mechanisms could be of physiological importance (Misiti *et al.* 1998). We thus explored the expression of 23 ER α co-regulators (Girault *et al.* 2003a) in the MVLN, CL6.8 and CL6.32 cell lines under exposure to E₂, OH-Tam or both E₂ and OH-Tam. No difference in expression was detected for 14 co-regulators between the MVLN and the OTR cell lines under E₂ or OH-Tam treatment (*EP300*, *CARM1*, *SRCAP*, *NCOA4*, *CREBBP*, *GCN5L2*, *SMARCA4*, *SMARCB1*, *NCOR1/N-CoR*, *HDAC1*,

SAP18, *HDAC3*, *MTA1* and *MTA2* genes; data not shown). The data presented in Table 2 demonstrate that E₂ treatment was able to downregulate the expression of several co-factors, either in all three cell lines or in at least one of the OTR cell lines (*NCOA2/TIF2/GRIPI*, *PCAF*, *RNF14/ARA54*, *NCOR2/SMRT*, *NCOA3/AIB1/ACTR*, *NCOA1/SRC1*, *TIF1* and *SAP30* genes). Interestingly, the expression of the *SIN3B* gene (coding for a co-repressor protein) was stimulated by OH-Tam in MVLN cells but not in the OTR cells (Table 2). This phenomenon was reinforced by the basal down-regulation of this gene in CL6.8 cells (FC, -2.77; untreated CL6.8 cells versus untreated MVLN cells; data not shown). In addition to *SIN3B*, the co-activator *EP300*, was the only gene that displayed a variation of expression at the basal level between the OTR cells and the MVLN cells (FC, +2.31 and +2.00, respectively, in CL6.8 and CL6.32 cells). Taken together, these findings highlight dysregulation of expression of several co-regulators between MVLN and the two OTR cell lines and suggest a possible involvement of such events in mediating the responses to OH-Tam and E₂.

Discussion

Because Tam-resistant breast tumors often display inappropriate responses such as altered gene expression and growth factor signaling, it is of clinical importance to delineate the molecular changes associated with the

Table 2 ER α transcriptional co-regulator expression in MVLN, CL6.32 and CL6.8 cell lines. Gene-expression variations were assessed on MVLN, CL6.8 and CL6.32 cells grown for 4 days under E₂ (1 nM) treatment, OH-Tam (200 nM) treatment, or both E₂ (1 nM) and OH-Tam (200 nM) treatment. The expression values presented are expressed in terms of fold change (FC) values (calculated by dividing the treated cell line gene-expression value by that obtained for the corresponding untreated cell line) and represent means from at least two independent RTQ-PCR measurements. Gene-expression variation was considered to be significant for FC ≥ 2 or FC ≤ -2 . Upregulations are indicated in bold and downregulations are underlined.

Gene name	Symbol	Accession no.	FC value					
			MVLN			CL6.32		
			E ₂	OH-Tam	E ₂ +OH-Tam	E ₂	OH-Tam	E ₂ +OH-Tam
Nuclear receptor co-activator 2	NCOA2	NM_006540	-2.30	-1.16	1.01	-2.31	-1.49	-1.93
p300/CBP-associated factor	PCAF	NM_003884	-10.11	-1.27	-1.20	-11.11	-1.92	-1.90
Ring finger protein 14	RNF14	NM_004290	-1.83	-1.07	1.26	-1.97	-1.25	-1.19
Nuclear receptor co-repressor 2	NCOR2	NM_006312	-1.75	1.15	1.21	-2.04	-1.33	-1.13
Nuclear receptor co-activator 3	NCOA3	NM_181659	1.01	1.62	1.66	-1.08	1.04	-1.16
Nuclear receptor co-activator 1	NCOA1	NM_003743	-1.06	1.42	1.77	-1.81	-1.24	-1.35
Transcriptional intermediary factor 1	TIF1	NM_003852	-1.67	-1.09	-1.16	-2.19	-1.71	-1.55
Sin3-associated polypeptide, 30kDa	SAP30	NM_003864	1.60	1.74	1.70	3.68	1.85	1.54
SIN3 homolog B	SIN3B	XM_050561	1.35	2.70	3.34	-1.28	-1.04	-1.04
						6.26	-1.59	3.07
						-2.43	-1.35	-1.10
						-15.44	-1.25	-1.47
						-2.28	-1.40	-1.04
						-3.65	-1.38	-1.32
						-2.26	-1.30	1.18
						-2.08	1.00	1.01
						-1.59	-1.04	1.06
						6.26	1.56	3.07
						-2.59	-1.16	-1.08

E₂ and OH-Tam responses in OTR models. Our work provides, for the first time, an extensive study of selective gene-expression patterns associated with both the E₂ and OH-Tam responses in the OTR CL6.8 and CL6.32 cell lines. These gene-expression variations might play a critical role in the development or the signature of OH-Tam resistance, and these genes could be new candidates as potential diagnostic/prognostic tools for OTR breast cancers. We decided that one of the criteria to select such candidates should be to consider genes whose expression variation was persistent, and which could thus reflect the physiological state of OTR breast tumors. For this reason, the pharmacologic treatment lasted 4 days.

Deprivation or blockade of estrogen action with Tam enhances the level of sensitivity to the stimulating action of E₂ on cell growth, and this event is associated with increased concentrations of ER α and ER-mediated events (Santen *et al.* 2001, Chan *et al.* 2002). Non-genomic actions of E₂ (Santen *et al.* 2004) but also ER-driven transcription events (Martin *et al.* 2003) have been identified as possible mechanisms. Our study provides evidence of a hyper-response to the growth-stimulatory action of E₂ in the OTR CL6.8 and CL6.32 cells. This event was associated with a lower expression level of ER α in CL6.8 and CL6.32 than in MVLN cells, demonstrating for the first time that a high level of ER α is not an absolute necessity for driving a hyper-response to E₂. We also observed that the hyper-response to E₂ in the OTR cell lines was associated at the gene-expression level with either the appearance of gene-expression variations that were not detected in MVLN cells or by the increase of the amplitude of the E₂ response measured in MVLN cells. The detection of a concomitant OH-Tam activity (antagonist or agonist effects) on the expression of the concerned genes suggests that these are later regulated by E₂ through ER-dependent mechanisms or pathways. Our work, however, did not permit us to distinguish between a direct or an indirect action of E₂, and future work will be necessary to decipher the exact molecular mechanism mediating the hyper-response to E₂ (e.g. by assessing estrogen responsiveness of promoter/reporter fusion constructs).

Investigation of the OH-Tam response in the OTR CL6.8 and CL6.32 cells demonstrated that this drug stimulates cell proliferation. This event was associated at the gene-expression level with both the loss of the antagonist activity of the drug and the appearance of a so-called estrogen-like signature. The genes identified in such regulations have been reported to encode proteins involved in the regulation of cell proliferation, DNA replication, genomic stability, DNA repair and

cell transformation and to be associated with the pathogenesis of or the clinical response to breast cancer. For example, *PTTG1/Securin* expression was recently identified as a prognostic marker of breast tumor aggressiveness (Solbach *et al.* 2004); high mRNA levels of *CCNE2* in breast tumors suggest that the encoded protein contributes to carcinogenesis (Payton *et al.* 2002); DNMT3B over-expression was associated with poor prognosis in breast tumor (Girault *et al.* 2003b); *NOV* encodes for a negative regulator of cell proliferation and its downregulation may be involved in malignant processes (Scholz *et al.* 1996); high *TGFB2* mRNA expression levels in OH-Tam-treated breast tumors were described as predictive of the clinical response (Brandt *et al.* 2003); loss of cyclin D2 expression could be associated with the evolution of breast cancer (Evron *et al.* 2001) and *EGR2/Krox-20* functions as a tumor suppressor and has growth-suppressive effects (Unoki & Nakamura 2001). Taken together, these findings suggest that the genes identified in our study might play a crucial role in OH-Tam-mediated physiological effects and in the development of OH-Tam resistance.

Besides its ability to promote G₀/G₁ cell-cycle arrest, Tam induces apoptosis in MCF-7 cells in a dose- and time-dependent manner (Zhang *et al.* 1999). Optimal concentrations demonstrated in studies *in vitro* usually occur at the micromolar level (10^{-5} and 10^{-6} M), but lower concentrations of Tam (10^{-7} and 10^{-8} M) can also trigger a slight but detectable induction of apoptosis (Zhang *et al.* 1999). In our study, we confirmed that OH-Tam was able to induce apoptosis in MVLN cells in a dose-dependent manner (data not shown). At 200 nM OH-Tam, the concentration at which the pro-apoptotic activity of the drug was not found to be maximal, we identified the upregulation of three pro-apoptotic genes (*TGFB2*, *TRADD* and *BIK*) that might be early-regulated genes involved in the OH-Tam-mediated induction of apoptosis (at higher concentrations of OH-Tam, however, other molecular mechanisms probably contribute to the activation of the cell-death process). Supporting this idea, the *BIK* gene was demonstrated recently to mediate fulvestrant-induced breast cancer cell apoptosis (Hur *et al.* 2004). Our study also demonstrated that the OTR CL6.8 and CL6.32 cells were refractory to the induction of apoptosis by OH-Tam. Again, this event could be correlated with specific expression patterns of genes coding for proteins involved in the regulation of apoptosis (*EGR2/KROX20*, *TGFB2*, *TRADD*, *TRAF1*, *TNFSF10/APO2L/TRAIL*, *BIK*, *BCL2*, *BBC3/PUMA* and *BIRC5/Survivin*), suggesting the importance of such regulation in the resistance to OH-Tam-induced

apoptosis developed by the CL6.8 and CL6.32 cell lines.

We next investigated two possible mechanisms that could contribute to the development of OH-Tam agonist activity and the hyper-response to E₂ in the OTR cells: (i) the level of MAPK activity and the phosphorylation status of ER α on Ser-118 and (ii) quantitative changes in the expression of transcriptional co-activators and co-repressors.

The activation of EGFR/HER2 and/or MAPK pathways have been demonstrated to contribute to phosphorylation of the AF-1 domain of ER α , leading to ligand-independent ER transcription (Kato *et al.* 1995), Tam agonist activity (Kato *et al.* 1995) and hypersensitivity to E₂ (Yue *et al.* 2002, Martin *et al.* 2003, Berstein *et al.* 2004). Additional studies suggest that in the context of hypersensitivity to E₂ (Jeng *et al.* 2000) or OH-Tam agonistic action (Shou *et al.* 2004) ER-mediated functions are involved in the elevation of HER2/EGFR and/or MAPK activity, suggesting a complex crosstalk between HER2/EGFR, MAPK, Tam resistance and hypersensitivity to E₂. In the present study, we found an increase in MAPK activity, an elevated phosphorylation status of ER α at Ser-118 and over-expression of several genes coding for EGFR and numerous ErbB-specific ligands in the OTR CL6.8 and CL6.32 cells. These observations suggest the possible involvement of such events in the development of the agonist activity of OH-Tam and the hyper-response to E₂. It can be suggested that the autocrine release of ErbB-specific ligands proteins could promote the activation of the corresponding ErbB receptors and downstream activation of the MAPK pathway. Interestingly, when compared with CL6.32 cells, CL6.8 cells displayed a greater MAPK activity, a more-pronounced over-expression of gene members of the ErbB-driven pathway and a greater E₂ hyper-response and OH-Tam agonist activity at the gene-expression level. Taken together, these observations suggest that MAPK activation, hyper-response to E₂, agonist activity of OH-Tam and possible autocrine control might be linked. The precise mechanisms for this interplay are, however, currently unknown, and future work will be necessary to decipher this possible complex crosstalk.

Several studies have suggested that changes in expression levels of cofactors might contribute to enhanced E₂-stimulated transcription or OH-Tam agonist activity (Smith *et al.* 1997, Shang & Brown 2002). Our study allowed the identification of several differences in ER α transcriptional co-regulator expression between MVLN and OTR cells, suggesting the involvement of such events in the development of OH-Tam resistance

Figure 7 Summary of the gene-expression dysregulation associated with OH-Tam resistance and/or hyper-response to E₂ in OTR CL6.8 and CL6.32 cells.

and/or the hyper-response to E₂ in OTR cells. The most striking piece of data was observed with the *SIN3B* gene (coding for a transcriptional co-repressor), whose expression was induced under OH-Tam treatment in MVLN cells but not in the OTR cells. Interestingly, this phenomenon was also reinforced in CL6.8 cells by downregulation of the expression of this gene at the basal level. In cells where Tam acts mainly as an antagonist, Tam-bound ER α has been shown to recruit co-repressors (such as SMRT and NcoR) but not co-activators to target promoters (Shang *et al.* 2000). Thus, it can be suggested that the level of *SIN3B* expression contributes to dysregulation of the balance between co-activators and co-repressors in the OTR cells and is involved in the development of OH-Tam resistance.

In conclusion, the present study allowed the identification of specific gene-expression variations associated with OH-Tam resistance and hyper-response to E₂ in two OTR cells. This is summarized in Fig. 7. Interestingly, 86 and 90% of the gene-expression variations associated with the hyper-response to E₂ detected in CL6.8 and CL6.32 cells, respectively, were also associated with OH-Tam agonist activity, suggesting that these two events could be mechanistically linked. Supporting data demonstrated that long-term exposure of MCF-7 cells xenografts to Tam enhances sensitivity, both to E₂ and the estrogenic effects of Tam (Berstein *et al.* 2004). The elevated MAPK activity, the phosphorylation status of ER α on Ser-118

and/or the dysregulation in the balance of transcriptional co-regulators we identified in the OTR CL6.8 and CL6.32 cells could be part of the mechanisms contributing to the modified response of these cells to ER ligands. However, other mechanisms such as phosphorylation of ER α at Ser-305 (Michalides *et al.* 2004), activation of the phosphoinositide 3-kinase/Akt pathway or phosphorylation of ER α at Ser-167 (Campbell *et al.* 2001, Martin *et al.* 2003, Yue *et al.* 2003) could also be involved and remain to be investigated.

This work also underscores the complexity of OH-Tam resistance, especially the fact that the two OTR cell clones studied displayed molecular changes affecting a great number of genes with various cellular functions, illustrating that the development of OH-Tam resistance is a multifactorial process involving different molecular events, as observed by others (Brockdorff *et al.* 2003, Ring & Dowsett 2004). Interestingly, 56 and 70% of the gene-expression variations associated, respectively, with the hyper-response to E₂ and OH-Tam agonist activity were detected in both the CL6.8 and CL6.32 cells. This suggests that the two individual cell lines displaying a similar degree of relative resistance to OH-Tam and issued from the same selection process could develop different but also common stratagems to escape from OH-Tam action. The delineation of the signaling pathways detected in both OTR cells underlines the importance of such regulations in the acquisition or the signature of

OH-Tam resistance. Our work also provides evidence that ER α over-expression is not a pre-requisite for either induction of hyper-response to E₂ or development of OH-Tam resistance. Interestingly, we observed that the level of ER β expression was low and did not display any variation between MVLN cells and the OTR cells. This suggests that ER β expression does not seem to be associated with progression from the OH-Tam-sensitive state to the OH-Tam-resistant and E₂-hyper-responsive states. In summary, this study provides new information on the molecular events subverting the cellular survival mechanisms associated with the development of OH-Tam resistance and could allow identification of potential molecular targets for diagnosis or therapy.

Acknowledgments

We thank Dr S L Salhi for editing the manuscript. We are grateful to Dr N Mechti for helpful discussions. We also thank Dr P Martineau and Dr C Theillet for critical comments, Dr C Duperrey for help with the FACS experiments and Miss C Saudray for technical assistance. This work was supported by grants from the Ligue Nationale Contre le Cancer and the Groupement des Entreprises Françaises dans la Lutte Contre le Cancer. J A V was supported by a scholarship from the Ligue Nationale Contre le Cancer (France). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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