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The antiestrogens 4-hydroxytamoxifen and fulvestrant are inhibitors of oncogenic factor Y-box binding protein-1 expression in breast cancer cells

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Summary

Purpose: The cold-shock protein Y-box binding protein-1 (YB-1) is associated with more frequent relapse and higher aggressiveness in breast cancer and, notably, is a client protein of estrogen receptor α (ERα). Thus, the authors hypothesized that endocrine therapy using the antiestrogens 4-hydroxytamoxifen (4-OHT) and fulvestrant (FUL) may affect YB-1 expression. *Materials and Methods:* YB-1 localization in the breast cancer cell line MCF-7 was determined by fluorescence microscopy and GST-ERα pull-down assay. Modulation of YB-1 expression in the presence of 4-OHT and FUL was determined by quantitative RT-PCR as well as by Western blot analysis. *Results:* YB-1 is primary localized in the perinuclear cytoplasm of MCF-7 cells. YB-1 binds directly to recombinant GST-ERα fusion protein as shown by pull-down assay. Incubation experiments with 4-OHT and FUL demonstrated a strong time- and dose-dependent suppression of YB-1 expression by these antiestrogens. Inhibitory effects were assessed on the level of YB-1 mRNA as well as on the level of YB-1 protein. *Conclusion:* The data presented here suggest that 4-OHT and FUL therapy targets both proliferative ERα as well as pro-oncogenic YB-1. Thus, 4-OHT's and FUL's anticancer efficacy may play a more global role in breast cancer progression control than originally thought.

Key words: Breast cancer; YB-1; ERα; Antiestrogen; 4-hydroxytamoxifen; Fulvestrant.

Introduction

The cold-shock protein Y-box binding protein-1 (YB-1) is highly conserved throughout evolution and exerts pleiotropic functions in the cell where it can be present in the cytoplasm as well as in the nucleus. Its ability to bind to DNA, especially to inverted CCAAT boxes (Y-boxes), as well as to RNA, allows YB-1 to regulate target gene expression on the level of transcription and translation [1]. Once accumulated in the nucleus, YB-1 is known to control cell fate via modulation of cyclins [2]. Moreover, YB-1 is associated with multidrug resistance due to expression of the drug-efflux pump P-glycoprotein (MDR1) [3]. Generally, correlation of a poor prognosis and increased YB-1 localization in the nucleus is seen in a variety of other human cancers like ovarian, brain, and lung cancer [4–6]. In breast cancer, molecular analysis partially delineate a mechanistic background for clinical studies, which revealed high YB-1 expression as a strong prognostic factor in breast cancer and correlated with more frequent relapse and higher aggressiveness [7–9].

There is an entire range of tumor-promoting receptors, such as receptors for members of the epidermal growth factor family, progesterone receptor, as well as the estrogen receptor α (ERα) which correlates with pro-oncogenic effects in cancer and which also seems to be regulated by the transcription factor YB-1 [10–12]. In a recent study on the role of YB-1 on transforming growth factor β (TGF β) functions in breast cancer progression, evidence suggests that antiestrogens may regulate the expression of YB-1. Thus, the present authors investigated the subcellular localization of YB-1 in the ERα positive breast cancer cell line MCF-7 and subsequently the impact of the antihormonal breast cancer drugs 4-hydroxytamoxifen (4-OHT) and fulvestrant (FUL) on the expression of YB-1.

Materials and Methods

The breast cancer cell lines MCF-7 and MDA-MB-231 were propagated in DMEM/F12 medium containing 4.5 g glucose/l, 5% fetal bovine serum (FBS), 1 mM sodium pyruvate, and 50 µg/ml gentamycin. Before their use in experiments, cells were maintained for one passage in the same medium as described above but containing 5% sulfatase and charcoal-treated FBS. Compounds 4-OHT and FUL were used in indicated concentrations.

MCF-7 cells were seeded on a slide, fixed with 80% acetone and incubated with a YB-1-directed antibody used in a dilution

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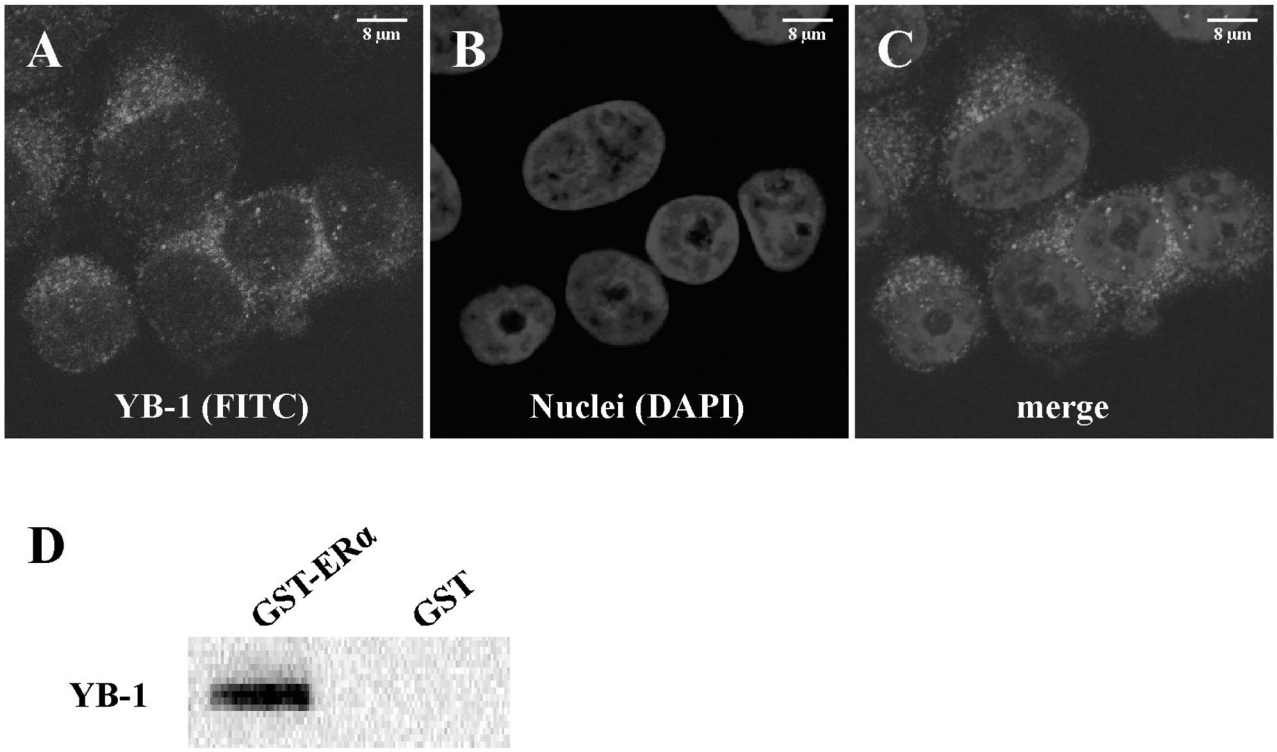

Figure 1. — Analysis of YB-1 localization in MCF-7 cells. (A-C) Fluorescence microscopy with MCF-7 cells was performed as described in the Materials and Methods section. Green: YB-1 (A), blue: nuclei (B), merge: overlay of both signals (C). Scale bar: 8 µm. (D) GST-ERα fusion protein (lane GST-ERα) and GST control protein (lane GST) were applied in pull-down assays utilizing MCF-7 cell lysates and introduced to YB-1-directed Western blot analysis.

of 1:500. Subsequently, the primary antibody was stained with a fluorescein-isothiocyanate (FITC)-conjugated secondary antibody . Nuclear staining was done by incubation with 4‘-6-diamidino-2-phenylindole. Cellular distribution was analysed with a fluorescence microscope.

A glutathione S-transferase (GST) fusion protein was generated by cloning of ERα cDNA in the expression vector pGEX-6P-1. Expression of GST-ERα fusion protein and GST control protein was induced in *E. coli* strain Rosetta 2. Cells were grown at 16°C overnight in the presence of 1 mM isopropyl β-D-1-thiogalactopyranoside . After cell disruption, proteins were purified using glutathione-sepharose beads according to the manufacturer´s instructions. GST-ERα fusion protein pull-down assays with MCF-7 cell lysate were performed as described elsewhere [13] using pull-down buffer (20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 0.5% Nonidet P40, 1 mM ethylenediaminetetraacetic acid, and Protease Inhibitor Cocktail Set III). Proteins binding to the glutathione-sepharose beads were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting.

For detection of YB-1 protein Western blotting was performed as described earlier [14]. Primary antibodies raised against YB-1 and glyceraldehyde 3-phosphate dehydrogenase were used in combination with the appropriate peroxidase-conjugated secondary antibody. Proteins were visualized using LumiGLO Reagent and Peroxide according to the manufacturer’s instructions.

For detection of YB-1 mRNA, total RNA was isolated utilizing the RNeasy Mini Kit according to the supplier’s instructions. 500 ng total RNA were used for reverse transcription with the Superscript III First-Strand Synthesis System according to the manufacturer’s instructions.

Target mRNA quantification was performed with the SYBR Green Dye on a Light Cycler using a serial dilution of target specific standard cDNAs. Specific primers were as follows: YB-1 se 5´-AAGTGATGGAGGGTGCTGAC-3´, YB-1 as 5´-TGACCTTGGGTCTCATCTCC-3´; β-glucuronidase (GUS) se 5´-GCTCATTTGGAATTTTGCCG-3´, and GUS as 5´-ATGCCCTTTTTATTCCCCAGC-3´.

Results

The oncogenic factor YB-1 interferes with functions in the cytoplasm and nucleus that are essential for tumor progression [1, 15]. To demonstrate the subcellular distribution of YB-1 in MCF-7 cells, specific detection of YB-1 (Figure 1A) and genomic DNA (Figure 1B) was performed and visualized by FITC staining and DAPI staining, respectively. YB-1 signals were granularly distributed in both in the cytoplasm as well as in the nucleus. Primarily, YB-1 was localized throughout the perinuclear cytoplasm (Figure 1C). Because some hints exist that YB-1 interferes with

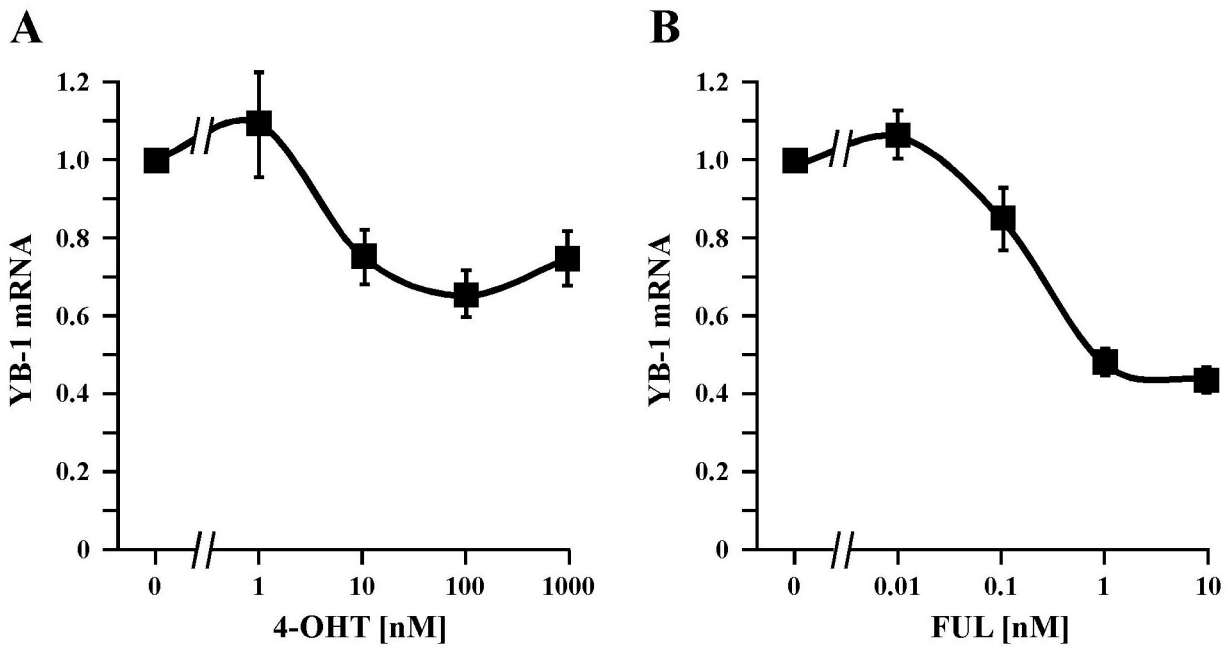

Figure 2. - Dose-dependent suppression of YB-1 by various concentrations of antiestrogens 4-OHT and FUL. (A) Relative 4-OHT-induced regulation of YB-1 mRNA levels over a concentration range of 1.0 nM to 1000 nM. (B) Relative FUL-induced regulation of YB-1 mRNA levels over a concentration range of 0.01 nM to 10 nM. After 72 hours of incubation mRNA levels were assessed by quantitative RT-PCR analysis and normalized to vehicle controls (0 nM=1.0).

ERα functionality [16, 17], and due to the co-localisation of both proteins within the same cell compartment of MCF-7 cells, a protein-protein interaction assay was performed (Figure 1D). The pull-down assay applying a GST-ERα fusion protein incubated with MCF-7 cell lysat demonstrated direct binding of YB-1 protein to recombinant GST-ERα fusion protein (lane GST-ERα). In contrast, control preparations utilizing GST protein missing the ERα sequence failed to bind to YB-1 protein (lane GST).

These data suggest that the regulation of ERα may also control the function of the ERα binding partner YB-1. Therefore, MCF-7 cells were incubated in the presence of the ERα antagonists 4-OHT and FUL and expression levels of YB-1 were examined. Both antiestrogens had an impact on the level of YB-1 mRNA transcription. 4-OHT (Figure 2A) as well as FUL (Figure 2B) provoked a strong dose-dependent suppression of basal YB-1 mRNA in MCF-7 cells. YB-1 modulation was sensitive to antiestrogen treatment over concentration ranges of 1.0 nM to 1000 nM (4-OHT) and 0.01 nM to 10 nM (FUL), respectively.

These observations were confirmed on the protein level. Again, MCF-7 cells incubated with 100 nM 4-OHT and 1.0 nM FUL showed a reduction of YB-1 over a period of 72 hours as shown by Western blot analysis (Figures 3A and B). Antiestrogen mediated suppression of YB-1 was time-dependent and after only 72 hours of incubation, YB-1 protein levels declined 1.1-fold (4-OHT) and 1.4-fold (FUL) compared to vehicle treated controls. Notably, this effect could not be detected in MDA-MB-231 cells missing the ERα protein (Figure 3C).

Discussion

Together with other hormone receptors, ERα is a pivotal factor in breast cancer progression control and accordingly the primary target for endocrine breast cancer therapy with antiestrogens. A few lines of evidence have recently demonstrated a regulatory link between ERα and YB-1 functionality. ERα seems to be an inhibitor of YB-1 expression and loss of ERα is associated with high levels of YB-1 expression as well as with relapse, metastasis, and poor survival in breast cancer patients [7, 9, 12]. Thus, the present authors hypothesized that efficacy of endocrine therapy with 4-OHT and FUL, the most commonly used antiestrogens [18], is partially caused by the suppression of oncogenic YB-1 properties.

The present study presented here verified the direct bind-

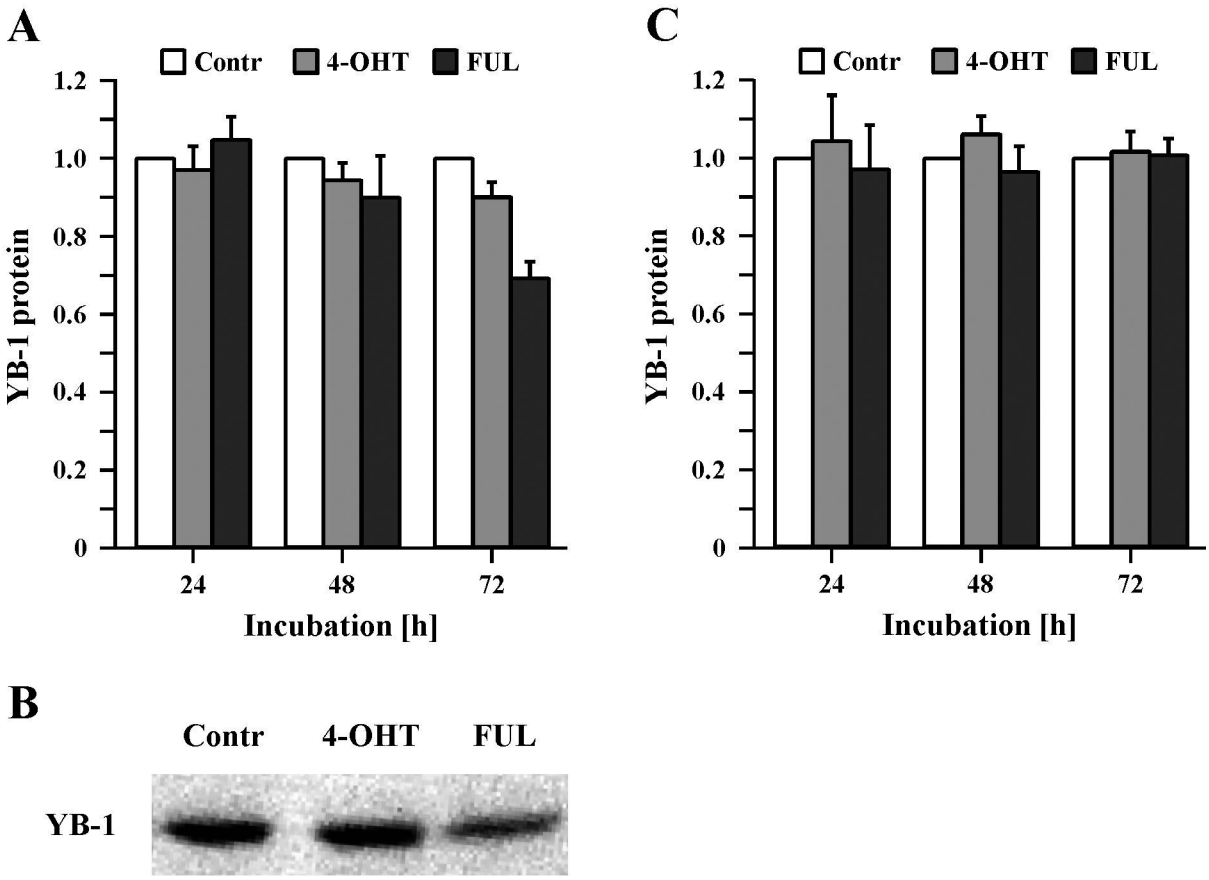

Figure 3. — Time-dependent suppression of YB-1 in the presence of antiestrogens 4-OHT and FUL. (A) Relative antiestrogen-induced YB-1 levels in ERα-positive MCF-7 cells over a period of 72 hours. (B) YB-1-directed Western blot analysis after 72 hours of incubation. (C) Relative antiestrogen-induced YB-1 levels in ERα-negative MDA-MB-231 cells over a period of 72 hours. Western blot analysis were normalized to vehicle controls (Contr = 1.0).

ing of ERα to its client protein YB-1. Moreover, the present data demonstrated a strong time- and dose-dependent suppression of YB-1 expression by antiestrogens. This newly identified regulatory activity of 4-OHT and FUL take place on the level of mRNA as well as protein. In this regard, the present data indicated differences in FUL's and 4-OHT's YB-1 suppressor efficacy which may be explained by biochemical properties of ERα: the receptor exhibits two transcriptional activation functions (TAF-1 and TAF-2). The partial antagonist 4-OHT binds to TAF-2 exclusively, whereas the pure antagonist FUL is enabled to bind both TAF-1 and TAF-2 [19]. For this reason FUL might be more effective for inhibition of YB-1 expression than the partial antagonist 4-OHT. In this context, FUL reveals a 100-fold lower half maximal inhibitory concentration (IC50) than 4-OHT as well [20, 21].

ERα is primarily responsible for cell growth and development and therefore a key factor in breast cancer initiation and progression [22]. YB-1 controls a multitude of pro-oncogenic pathways including signaling cascades of cell transformation, treatment resistance, and cell motility [23]. The present data suggest that 4-OHT and FUL therapy targets both, proliferative ERα as well as pro-oncogenic and anti-therapeutic YB-1 activities. From there, 4-OHT's and FUL's anticancer efficacy may be more complex and may play a more global role in breast cancer progression control than originally assumed.

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