Sequential versus combined treatment of human breast cancer cells with antiestrogens and the vitamin D analogue EB1089 and evaluation of predictive markers for vitamin D treatment

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Summary
Development of resistance to antihormonal therapy is a major problem in the treatment of breast cancer patients. Metastatic tumors, which progress after a period of response to treatment, often respond to second line endocrine treatment, but eventually develop estrogen independent growth. Vitamin D analogues are promising new drugs, using alternative mechanisms to inhibit growth of breast cancer cells. The sensitivity to antiestrogens and vitamin D analogues has been proposed to be inverse, indicating that the sensitivity to vitamin D analogues might increase after development of antiestrogen resistance and vice versa. In this study, the inverse sensitivity between antiestrogens and the vitamin D analogue EB1089 was examined in antiestrogen and vitamin D resistant breast cancer cell lines. The majority of the investigated antiestrogen resistant cell lines had increased sensitivity to treatment with the vitamin D analogue EB1089. In addition, growth of a vitamin D resistant cell line was more inhibited by the pure antiestrogen ICI 182,780 than the growth of the parental cells, indicating that the compounds may be used sequentially. An association between the expression level of the vitamin D receptor (VDR) and EB1089 sensitivity was observed, suggesting that VDR is a possible predictive marker for response to vitamin D treatment. Valuation of Bcl-2 gene expression may be useful in combination with VDR to predict the outcome of treatment with EB1089. Furthermore, we observed a synergistic growth inhibition and an abrogated development of resistance upon combined treatment with ICI 182,780 and EB1089. Therefore, antiestrogens and vitamin D analogues may also be used as combined treatment for breast cancer patients in the future.

Introduction
Breast cancer is the most frequent cancer disease among women in the Western world. Every year the disease accounts for one third of the new female cancer incidences in the US, and the female lifetime risk of developing breast cancer approximates 13 percent [1]. The non-steroidal antiestrogen tamoxifen has been the leading drug for treatment of advanced breast cancer for over 30 years. Aromatase inhibitors have lately been shown to be superior to tamoxifen and will most likely become the primary choice for first line endocrine treatment of breast cancer patients [2, 3]. Expression of the estrogen receptor α (ERα) is routinely used as a predictive marker for response to endocrine treatment. Subdivision of ERα positive patients clearly shows that the response to tamoxifen or letrozole increases with increasing receptor expression [4, 5]. Upon development of antiestrogen resistance a total loss of ERα expression is rarely seen, but a decrease in the expression level is observed in patients [6]. This is also reflected by the decreased but not lost expression of ERα in the antiestrogen resistant cell lines in our cell model system [7]. These cell lines are still ER positive but show decreased sensitivity to antiestrogens. The definition...
of resistance must be ability of a tumor or a cell line to survive treatment although growth may be slower in the presence of the given compound. Therefore, the difficult task is to set a threshold for a predictive marker. Approximately 50–60 percentages of the ER positive patients benefit from treatment with tamoxifen [4, 8]. Following a period with good response to tamoxifen treatment, the tumors of the majority of the patients with metastatic disease progress. After development of resistance towards the primary agent, the tumors may respond to subsequent treatment with other antiestrogens or aromatase inhibitors. Such tumors eventually develop estrogen independent growth, and thus endocrine treatment becomes ineffective [9]. Vitamin D analogues are a promising new group of drugs using alternative, but not fully understood mechanisms in inhibition of breast cancer cell growth. Unfortunately, the active metabolite of vitamin D, 1α25(OH)2D3, causes hypercalcemia when used as systemic treatment [10]. Therefore, hundreds of 1α25(OH)2D3 analogues have been synthesized with the aim of finding a compound with reduced effect on calcium homeostasis. One of the promising new analogues is Seocalcitol/EB1089 synthesized by Leo Pharma, Ballerup, Denmark [11]. EB1089 has been tested in rat models, where oral administration resulted in significant regression of tumor progression. No effects were seen with the same concentrations of 1α25(OH)2D3 [12]. Furthermore, EB1089 has a very good toxicity profile (reviewed in [11]). Phase 2 studies in humans raised the question whether vitamin D receptor (VDR) could be used as a marker for treatment with EB1089, as VDR was detected in all responders while some of the non-responders had undetectable expression levels of VDR [13].

The existence of an inverse relationship between sensitivity to antiestrogens and vitamin D analogues as well as between expression of VDR and ERα has been proposed [14]. This hypothesis is supported by increased VDR expression and growth inhibitory effect of vitamin D compounds in antiestrogen resistant breast cancer cell lines [14, 15]. Similarly, an increased ERα expression and sensitivity to antiestrogen in vitamin D resistant cell lines has been observed [14, 16]. In this study, we expand our studies of sensitivity towards EB1089 to a panel of six antiestrogen resistant breast cancer cell lines developed in our laboratory and to investigation of the value of VDR and Bcl-2 as a predictive markers. In addition, we investigate a vitamin D resistant and a vitamin D hypo sensitive breast cancer cell line [16, 17] for ERα content and sensitivity towards treatment with the antiestrogens ICI 182,780 and tamoxifen, as well as VDR and Bcl-2 content and EB1089 sensitivity.

The expression level of the antiapoptotic protein Bcl-2 has been implied to play an important role in sensitivity to vitamin D treatment [18]. In concordance, we have earlier found that two antiestrogen resistant breast cancer cell lines with reduced expression of the antiapoptotic protein Bcl-2 displayed increased sensitivity to the growth inhibitory effect of EB1089 [15]. With the aim of identifying possible predictive markers for response to vitamin D treatment, the association between expression of VDR or Bcl-2 and sensitivity to EB1089 was analyzed in six antiestrogen resistant cell lines, one vitamin D resistant cell line and one vitamin D hypo sensitive breast cancer cell line and the respective parental cell lines. Furthermore, we evaluate the effect of combined treatment with antiestrogens and EB1089 on growth and development of resistance.

Materials and methods

Cell lines and culture conditions

The human breast cancer cell line MCF-7 was originally obtained from the Breast Cancer Task Force Cell Culture Bank, Mason Research Institute (Worcester, MA). The original MCF-7 cell line has been adapted to grow in medium with low serum concentration and is designated MCF-7/S0.5 [19]. MCF-7/S0.5 cells were maintained in phenol red free DMEM/F12 (1:1) growth medium (Life Technologies, Bethesda, MD) supplemented with 1% heat inactivated Fetal Calf Serum (FCS, Sigma-Aldrich, St. Louis, Missouri), 6 ng/ml insulin (Novo Nordisk, Copenhagen, Denmark) and 2.5 mM glutamax (Life Technologies) in T-25 flasks (Nunc, Roskilde, Denmark). Growth medium was renewed every 2nd or 3rd day, and the cells were subcultivated by trypsinization once a week with a split ratio of approximately 20.

Antiestrogen resistant cell lines were established from MCF-7/S0.5 cells. The antiestrogen resistant cell lines MCF-7/TamR-1 [20] MCF-7/182R-1, MCF-7/182R-6, MCF-7/182R-7, MCF-7/164R-5 [21] and MCF-7/RU58R-1 were selected for their ability to grow in the presence of 10−6 M tamoxifen, 10−7 M ICI 182,780, 10−7 M ICI 164,384 or 10−9 M RU58,668, respectively. The resistant cell lines are cultured routinely with addition of their respective