

Anti-oestrogen resistant human breast cancer cell lines are more sensitive towards treatment with the vitamin D analogue EB1089 than parent MCF-7 cells

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Summary Most breast cancer patients treated with anti-oestrogens will eventually develop resistance towards treatment. Therefore it is important to find new therapeutic agents effective for treatment of patients relapsing on anti-oestrogen. The vitamin D analogue EB1089 (Seocalcitol™) is a promising new agent for treatment of breast cancer patients with advanced disease, and in this study we show that two different anti-oestrogen-resistant human breast cancer cell lines are more sensitive towards treatment with EB1089, than the parent MCF-7 cell line. The two resistant cell lines both express a lower content of the anti-apoptotic protein Bcl-2, and we suggest that this may explain the higher sensitivity towards EB1089. The importance of Bcl-2 in response to EB1089 is supported by our observation that oestradiol abrogates the effect of EB1089 in cell lines which increase Bcl-2 in response to oestradiol treatment. Overall these results indicate that treatment with Seocalcitol™ may prove effective when patients become refractory to anti-oestrogen therapy, and that Bcl-2 may be used as a predictive marker. © 2001 Cancer Research Campaign <http://www.bjcancer.com>

Keywords: MCF-7; anti-oestrogen resistance; ER α ; Bcl-2; vitamin D analogue, EB1089

Tamoxifen, a non-steroidal oestrogen antagonist, is the most widely used anti-oestrogen in endocrine treatment of advanced breast cancer. About 60% of the patients with ER-positive primary tumours respond to tamoxifen therapy for advanced disease (Osborne et al, 1980). Unfortunately, all patients will eventually develop resistance to tamoxifen treatment, but several patients will respond to second and third line of hormone therapy, such as progestins, aromatase inhibitors or the steroidal and pure antagonistic anti-oestrogen ICI 182 780 (Faslodex™) (Howell et al, 1995). Tamoxifen has both antagonistic and agonistic activities and the agonistic effects may be responsible for some cases of tamoxifen resistance (Howell et al, 1990). However, treatment with ICI 182 780 also result in outgrowth of resistant cells both in vitro, in cell cultures (Lykkesfeldt et al, 1995; Brüner et al, 1997), and in model studies in nude mice (Osborne et al, 1995). Development of resistance towards anti-oestrogens is thus a major clinical problem. It is therefore of extreme importance to obtain a detailed knowledge of anti-oestrogen resistance to further improve treatment of patients relapsing on anti-oestrogen treatment. The vitamin D analogue EB1089 is an interesting new and promising anti-cancer drug detailed reviewed in Hansen et al (2000). It has been shown to be highly antiproliferative and to induce apoptosis in breast cancer cells both in vitro and in vivo without causing hypocalcaemia (Mathiasen et al, 1993; Love et al, 1996; Xie et al, 1997, 1999; Gulliford et al, 1998; James et al, 1998; El Abdaimi et al, 1999; Mathiasen et al, 1999). EB1089 has also been shown to inhibit tumour growth of a newly established ICI 182 780 resistant MCF-7 subline in nude mice (Nolan et al, 1998). In this study, we

have used two well characterized anti-oestrogen resistant cell lines, MCF-7/TAM^R-1 and MCF-7/182^R-6 (Lykkesfeldt and Briand, 1986; Lykkesfeldt and Sørensen, 1992; Wiseman et al, 1993; Lykkesfeldt et al, 1994, 1995; Larsen et al, 1997, 1999; Madsen et al, 1997; Jensen et al, 1999), and observed that these resistant cell lines responded to treatment with EB1089. In fact, they were more sensitive than the parent MCF-7 cells. The increased sensitivity to EB1089 treatment was found to be associated with a reduced level of the anti-apoptotic protein Bcl-2 in the resistant cells.

MATERIALS AND METHODS

Cell cultures and growth experiments

The MCF-7 cell line was originally obtained from the Breast Cancer Task Force Cell Culture Bank, Mason Research Institute (Worcester, MA). The MCF-7 cells are routinely propagated in growth medium consisting of phenol red free DME/F12 (1:1) + 2.5 mM Glutamax (Life Technologies, Roskilde, Denmark) supplemented with 1% fetal calf serum (FCS) and 6 ng ml⁻¹ insulin (Novo-Nordic, Copenhagen, Denmark) (Lykkesfeldt et al, 1995). The tamoxifen resistant cell line MCF-7/TAM^R-1 and the ICI 182 780 resistant cell line MCF-7/182^R-6 were established as described earlier (Lykkesfeldt and Briand, 1986; Lykkesfeldt et al, 1995). Cultures used for growth experiments were seeded, 10⁴ cells/cm², in 2 cm² multiwell dishes (Nunc, Roskilde, Denmark). 2 days after seeding experimental media were added. Stock solution of 10⁻² M oestradiol (Sigma, St Louis, MO) in 96% ethanol and stock solution of 4 × 10⁻³ M EB1089 in 99% isopropanol (LEO Pharmaceuticals, Ballerup, Denmark) were stored in -20°C freezer. For growth experiments EB1089 were further diluted in isopropanol. EB1089 and oestradiol were added to the experimental media at the time of change of media, which

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was every 2nd or 3rd day during the experiments. 4 wells were used for each cell number determination, which occurred at day 6 by counting in a Bürker-Türk chamber. Growth experiments were performed 3 times on independent cell lines.

Western analysis

MCF-7, MCF-7/TAM^R-1 and MCF-7/182^R-6 cell lines were grown 1 week in control growth medium (1% FCS) before onset of the experiments. Cells were seeded in T25 flasks (Nunc) in control growth medium at a density of 2×10^5 cells/T25 flask. Experimental media were added 2 days after seeding, and medium was renewed every 2nd or 3rd day. After growth for 6 days in experimental medium, the cells were washed with PBS and harvested in RIPA-buffer (Larsen et al, 1999). 30 µg of total protein per sample (determined by Bio-Rad protein assay kit, Munich, Germany) were run on 15% SDS-PAGE gels under reducing conditions. The proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA) by semi-dry electroblotting. Immunoassaying was done using a primary mouse monoclonal antibody against human Bcl-2 (Transduction Laboratories, Lexington, KY), a primary mouse monoclonal anti-human ER α antibody (1D5; DAKO, Glostrup, Denmark) and a secondary rabbit-anti-mouse horseradish peroxidase-conjugated antibody (P0260; DAKO). The blots were stripped and immunoassayed with a primary mouse monoclonal antibody against human keratin 7 (kindly provided by Dr Jiri Lukas) as a control for differences in protein loading. The enhanced chemiluminescence (ECL^{PLUS}) detection system (Amersham Pharmacia Biotech, Hørsholm, Denmark) was used for visualization of the proteins according to the manufacturer's instructions. The blots were scanned and quantification was done with ImageQuant software (Amersham Pharmacia Biotech). The Western analysis was repeated 3 times on independent protein preparations and the results were reproducible.

RESULTS

Anti-oestrogen-resistant cell lines are more sensitive towards treatment with the vitamin D analogue EB1089

Dose-response experiments with MCF-7 cells and the two resistant cell lines clearly show that EB1089 are more potent for inhibition of growth of the resistant cell lines (Figure 1). The IC₅₀ values for MCF-7/TAM^R-1 and MCF-7/182^R-6 are 1.5×10^{-9} M, and 1.5×10^{-8} M for MCF-7, showing that EB1089 exerts a 10 times more potent growth inhibitory effect on the anti-oestrogen resistant cell lines.

The response rate towards treatment with EB1089 correlates with the Bcl-2 expression level

The response towards EB1089 has recently been shown to correlate with the Bcl-2 protein level in a MCF-7 subline (Mathiasen et al, 1999). We therefore tested whether there were any differences in the basal Bcl-2 expression level among the cell lines, and whether EB1089 had effects on Bcl-2 expression. Figure 2 shows that the basal expression level was 6–8 fold higher in MCF-7 cells compared to both anti-oestrogen resistant cell lines. The MCF-7/TAM^R-1 and MCF-7/182^R-6 resistant sublines have almost the same basal Bcl-2 expression level. EB1089 alone did not show any

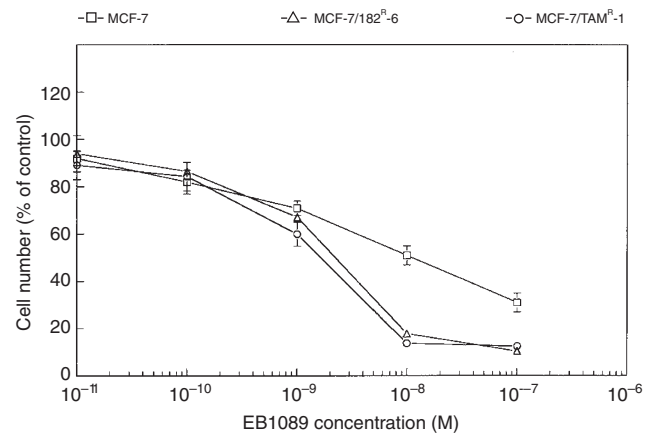


Figure 1 Dose-response curves for effect of EB1089 on cell proliferation of MCF-7, MCF-7/TAM^R-1 and MCF-7/182^R-6 cells. MCF-7, MCF-7/TAM^R-1 and MCF-7/182^R-6 cells were grown 1 week in 1% FCS before onset of experiment. Cells (2×10^4) were seeded in each 2 cm² multi-well, and 2 days after seeding experimental media with the indicated concentrations of EB1089 were added. Medium was renewed every 2nd or 3rd day. Cell numbers in quadruplicate wells were determined 6 days after addition of experimental medium. Mean values (expressed as percentage of the corresponding control culture without EB1089) and SDs are shown. SDs are not displayed when lower than 3%. The results are from one representative experiment out of 3 independent experiments with reproducible results

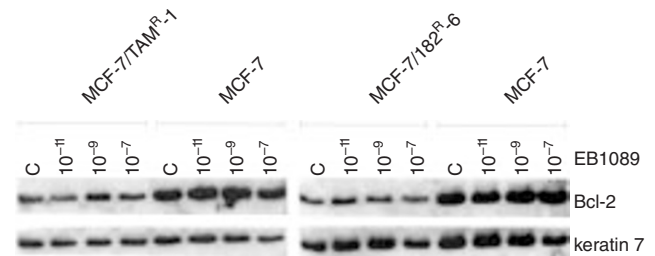


Figure 2 Western blot (ECL) showing the expression of Bcl-2 in MCF-7, MCF-7/TAM^R-1 and MCF-7/182^R-6 cells. Bcl-2 expression was determined after 6 days of hormonal treatment. Total cell extracts (30 µg of protein) from the 3 cell lines were loaded in each lane on a 15% SDS-PAGE gel, electroblotted, and immunostained with a primary mouse monoclonal antibody against human Bcl-2. The blot was stripped and immunostained with a primary mouse monoclonal antibody against human Keratin 7 as a control for protein loading. The immunocomplexes were visualized using the ECL^{PLUS} detection system (Amersham Pharmacia Biotech). C: Control 1% FCS; 10⁻¹¹ M, 10⁻⁹ M and 10⁻⁷ M EB1089. Representative results from one of three independent experiments are shown

regulatory effects on Bcl-2 protein expression, in the concentration range 10⁻¹¹ M to 10⁻⁷ M (Figure 2).

Oestradiol reduces the growth inhibitory response to EB1089 treatment concomitant with an increase in Bcl-2 expression

It has been shown that oestradiol induces Bcl-2 expression at the transcriptional level in MCF-7 cells, and protects the cells from induction of apoptosis (Dong et al, 1999; Mathiasen et al, 1999; Perillo et al, 2000). We therefore tested whether oestradiol could abolish or reduce the growth inhibitory effect of EB1089. Oestradiol almost completely abolished the growth inhibitory effect of EB1089 on MCF-7, exerted a partial abrogation in

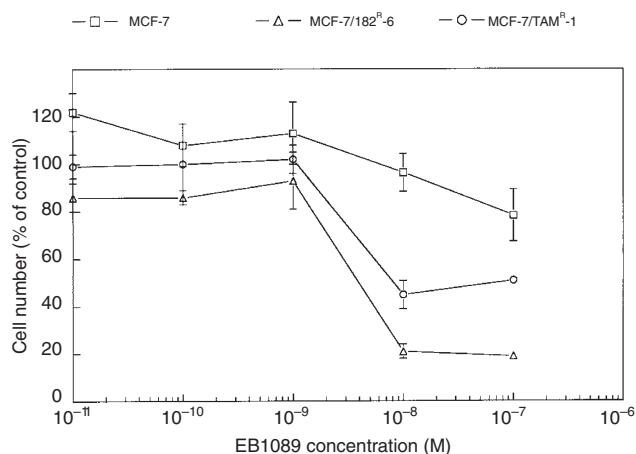


Figure 3 Dose-response curves for effect of EB1089 plus oestradiol on cell proliferation of MCF-7, MCF-7/TAM^R-1 and MCF-7/182^R-6 cells. MCF-7, MCF-7/TAM^R-1 and MCF-7/182^R-6 cells were grown 1 week in 1% FCS before onset of experiment. Cells (2×10^4) were seeded in each 2 cm² multi-well, and 2 days after seeding experimental media with the indicated concentrations of EB1089 plus 10^{-9} M oestradiol were added. Medium was renewed every 2nd or 3rd day. Cell numbers in quadruplicate wells were determined 6 days after addition of experimental medium. Mean values (expressed as percentage of the corresponding control culture without hormone) and SDs are shown. SDs are not displayed when lower than 3%. The results are from one representative experiment out of 3 independent experiments with reproducible results

MCF-7/TAM^R-1, and had no effect on MCF-7/182^R-6 cells (Figure 3 in comparison with Figure 1).

As observed in Figure 3, oestradiol did not abrogate the effect of EB1089 to the same extent in the 3 cell lines, and we tested whether the induction of Bcl-2 by oestradiol alone or in combination with EB1089 was different among the cell lines. Oestradiol alone induced the Bcl-2 protein expression 1.5–1.7 fold in MCF-7, 2.5–3.4 fold in MCF-7/TAM^R-1, and 2–2.5 fold in MCF-7/182^R-6 cells (Figure 4A). Although, the relative fold induction of Bcl-2 by oestradiol in the resistant cell lines was higher than in MCF-7 cells, the level never reached the same high amount as found in MCF-7 cells (Figure 4A). The induction of Bcl-2 by oestradiol in combination with EB1089 was 1.3–1.6 fold in MCF-7 and 1.5–2 fold in MCF-7/TAM^R-1, whereas in MCF-7/182^R-6 cells no induction could be detected (Figure 4B).

EB1089 down-regulates the oestrogen receptor α

We speculated whether the ER α level could be important for the reduced oestradiol induction of Bcl-2 in combination with EB1089. Figure 4C shows that the ER α is down regulated by 10^{-7} M EB1089, the same concentration reducing the Bcl-2 induction in combination with oestradiol observed in the anti-oestrogen resistant cell lines (Figure 4B, lanes 6 and 9). In MCF-7 cells the ER α level is reduced about 40–50% by EB1089, in MCF-7/TAM^R-1 cells there is a 65–70% reduction, and in MCF-7/182^R-6 the ER α is completely down-regulated, suggesting that the reduced induction of Bcl-2 by oestradiol in combination with EB1089 is due to the down-regulation of ER α by EB1089.

DISCUSSION

The data presented in this work show that anti-oestrogen-resistant cell lines are 10 times more sensitive towards treatment with the vitamin D analogue EB1089 than parent MCF-7 cells. We also

show that the resistant cell lines have a lower basal Bcl-2 expression level than parent MCF-7 cells. This is in concert with our previous observation that all the resistant cell lines have a lower basal expression level of both ER α and oestrogen-regulated genes. Furthermore, they all lack PR expression (Lykkesfeldt et al, 1994, 1995). Several clinical studies have shown that the Bcl-2 expression in breast tumours is strongly correlated to both ER α and PR expression, and Bcl-2 expression is an indicator of a favourable outcome following endocrine treatment (Gee et al, 1994; Lipponen et al, 1995; Silvestrini et al, 1996; Elledge et al, 1997; Keen et al, 1997; Kobayashi et al, 1997; Olopade et al, 1997; Holmqvist et al, 1999). The decreased Bcl-2 expression associated with decreased ER α expression, and lack of response to anti-oestrogens observed in the resistant cell lines is in concert with the above-mentioned clinical studies.

The ratio between the pro-apoptotic and anti-apoptotic members in the Bcl-2-family has been shown to dictate the sensitivity or resistance of cells to a wide variety of apoptotic stimuli (Oltvai and Korsmeyer, 1994). In the two resistant cell lines the basal expression level of Bcl-2 is 6–8 fold lower than in the MCF-7 cell line, and consequently the critical ratio may be shifted in the pro-apoptotic direction rendering these cells more sensitive to apoptosis induced by EB1089, compared to parent MCF-7 cells. In MCF-7 cells, the critical ratio of Bcl-2 required to inhibit apoptosis may be reached at a 1.3–1.6 fold induction by oestradiol as indicated by the ability of oestradiol to abrogate the inhibitory effect of EB1089 in these cells. The induction of Bcl-2 with oestradiol in combination with EB1089 is 2.5–3.4 fold in MCF-7/TAM^R-1 cells. However, this Bcl-2 level is significantly lower than in oestradiol-treated MCF-7 cells and may, therefore, not be sufficient to protect the cells completely against the EB1089-induced apoptotic stimuli. Further support for the association between the Bcl-2 expression level and the effect of EB1089 is obtained by our results with the MCF-7/182^R-6 cells, in which oestradiol in combination with EB1089 neither induces Bcl-2 expression nor abolishes growth inhibition. A similar association between the Bcl-2 expression level and the response to EB1089 treatment has recently been observed in a different model system with Bcl-2-transfected cell lines (Mathiasen et al, 1999). The reduced induction of Bcl-2 by oestradiol in combination with EB1089 can be explained by the EB1089 mediated down-regulation of the ER α which is required for oestradiol induction of Bcl-2. EB1089-mediated down-regulation of ER α has also been reported by others (Simboli et al, 1997; Swami et al, 2000).

We did not observe any EB1089 regulation of the Bcl-2 expression (Figure 2), indicating that EB1089 does not mediate its effect via a down-regulation of this anti-apoptotic protein in our model system. This is in contrast to what has been shown by others, who found an EB1089 mediated down-regulation of Bcl-2 (Simboli et al, 1997). However, not only Bcl-2 but also IGF-binding proteins (IGFBPs) may be important for growth inhibition.

It has been reported that both EB1089 and the anti-oestrogens tamoxifen and ICI 182 780 up-regulate IGFBP-3 and IGFBP-5 in MCF-7 cells, and suggested that both EB1089 and the anti-oestrogens mediate their growth inhibitory effect via up-regulation of IGFBP-3 and IGFBP-5 (Rozen et al, 1997; Colston et al, 1998), which are known to induce apoptosis in MCF-7 cells (Huynh et al, 1996a, 1996b). Our data showing the lack of cross-resistance between tamoxifen, ICI 182 780 and EB1089 reported in this study and in Lykkesfeldt et al (1995) indicate that different mechanisms are responsible for growth inhibition mediated by these

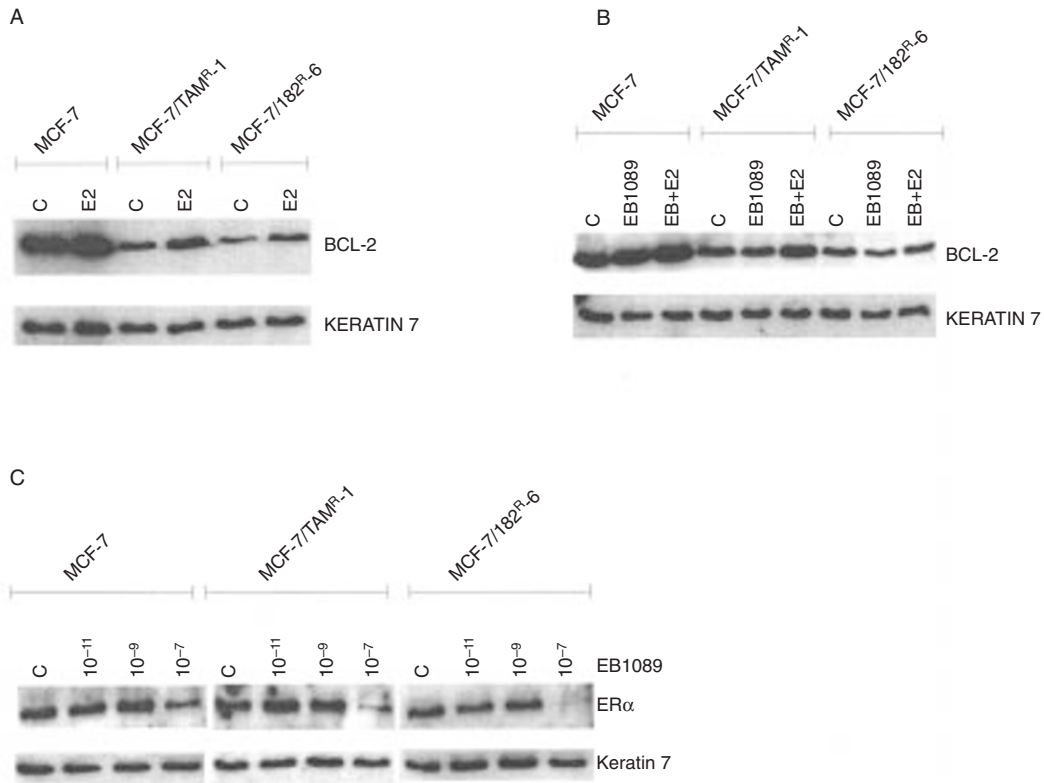


Figure 4 Western blot (ECL) showing Bcl-2 and ER α expression in MCF-7, MCF-7/TAM^R-1 and MCF-7/182^R-6 cells treated with EB 1089 and oestradiol. Bcl-2 and ER α expression was determined after 6 days of hormonal treatment. Total cell extracts (30 μ g of protein) were loaded in each lane on a 15% SDS-PAGE gel, electroblotted, and probed with a primary mouse monoclonal antibody against human Bcl-2, or a primary mouse monoclonal anti-human ER α antibody. The blots were stripped and immunostained with a primary mouse monoclonal antibody against human keratin 7 as a control for protein loading. The immunocomplexes were visualized using the ECL^{PLUS} detection system (Amersham Pharmacia Biotech). Expression of Bcl-2. (A) C: Control 1% FCS; E2: 10⁻⁹ M 17- β -oestradiol. Expression of Bcl-2, (B) C: Control 1% FCS, EB: 10⁻⁷ M EB1089 and EB+E2: 10⁻⁷ M EB1089 plus 10⁻⁹ M 17- β -oestradiol. Expression of ER α . (C) C: Control 1% FCS; 10⁻¹¹ M, 10⁻⁹ M and 10⁻⁷ M EB 1089. Representative results from one of the three independent experiments are shown

drugs. At present, we are exploring whether IGFBP-3 and IGFBP-5 are involved in growth inhibition. In accordance with our assumption, JoEllen Welsh has also suggested that vitamin D₃ compounds and anti-oestrogens trigger growth arrest and apoptosis in MCF-7 cells by distinct mechanisms (Welsh, 1997). The JoEllen Welsh group has shown that MCF-7 cells resistant towards vitamin D₃ are sensitive to tamoxifen and ICI 182 780 treatment (Welsh, 1994; Narvaez and Welsh, 1997; Nolan et al, 1998).

Importantly, our data strongly suggest the Bcl-2 protein is not involved in development of resistance towards anti-oestrogens, due to the 6–8 fold lower Bcl-2 expression compared to parent MCF-7 cells. Furthermore our data demonstrate that acquired resistance towards anti-oestrogens does not render the cells multi-resistant to induction of apoptosis by other agents like EB1089.

Our experiments indicate that there may be a positive correlation between the degree of hormone independence and the response rate toward treatment with EB1089. MCF-7 cells are totally oestrogen dependent, MCF-7/TAM^R-1 are slightly oestrogen independent and MCF-7/182^R-6 are almost completely oestrogen independent for growth in vitro and in vivo (Lykkesfeldt et al, 1994; Jensen et al, 1999; Larsen et al, 1999). We have shown here for the first time, that it is favourable, with respect to treatment with

EB1089, that the anti-oestrogen-resistant cells have progressed into a less hormone-dependent state, with a reduced expression of oestrogen-regulated genes e.g. Bcl-2, due to a lower ER α content.

Furthermore, the fact that both anti-oestrogen-resistant cell lines are more sensitive towards EB1089 than the parent MCF-7 cells looks promising for future treatment strategies. Clinical studies using EB1089 as treatment of different cancer types have demonstrated that the drug has no severe hypocalcaemia effects in humans (Gulliford et al, 1998; El Abdaimi et al, 1999). It has recently been reported that the development of SeocalcitolTM is active as an anti-cancer drug (Hansen et al, 2000). We therefore suggest that SeocalcitolTM (EB1089) may prove useful in treatment of breast cancer patients who have relapsed on anti-oestrogen treatment, and that the Bcl-2 expression level may be used to select the patients most likely to respond to treatment with SeocalcitolTM.

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