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Calcitriol and its analogues enhance the antiproliferative activity of gefitinib in breast cancer cells

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ABSTRACT

Coexpression of EGFR and HER2 has been associated with poor disease outcome, high rates of metastasis and resistance to conventional treatments in breast cancer. Gefitinib, a tyrosine kinase inhibitor, reduces both cell proliferation and tumor growth of breast cancer cells expressing EGFR and/or HER2. On the other hand, calcitriol and some of its synthetic analogs are important antineoplastic agents in different breast cancer subtypes. Herein, we evaluated the effects of the combined treatment of gefitinib with calcitriol or its analogs on cell proliferation in breast cancer cells.

The presence of EGFR, HER2 and vitamin D receptor were evaluated by Western blot in two established breast cancer cell lines: SUM-229PE, SKBR3 and a primary breast cancer-derived cell line. The antiproliferative effects of gefitinib alone or in combination with calcitriol and its analogs, calcipotriol and EB1089, were assessed by growth assay using a DNA content-based method. Inhibitory concentrations on cell proliferation were calculated by non-linear regression analysis using sigmoidal fitting of dose-response curves. Pharmacological effects of the drug combinations were calculated by the Chou–Talalay method. Phosphorylation of ERK1/2 MAPK was evaluated by Western blot. Gene expression of EGFR, HER2 and BIM was assessed by real time PCR. BIM protein levels were analyzed in cells by flow cytometry. The effects of the drugs alone or combinated on cell cycle phases were determined using propidium iodide. Apoptosis was evaluated by detection of subG1 peak and determination of active caspase 3 by flow cytometry.

Gefitinib, calcitriol, calcipotriol and EB1089 inhibited cell proliferation in a dose dependent manner by reducing percentages of cells in G2/M phase. The combinations of gefitinib with calcitriol or its analogs were more effective to inhibit cell growth than each compound alone in all breast cancer cells studied. The gene expression of EGFR and HER2 was downregulated and not affected, respectively, by the combined treatment. Furthermore, phosphorylation of ERK 1/2 was inhibited a greater extent in co-treated cells than in the cells treated with alone compounds. The combination of gefitinib with calcitriol or their synthetic analogs induced apoptosis in SUM-229PE cells, this was shown by the significant upregulation of BIM protein levels, higher percentages of cells in subG1 peak and increase of caspase 3-positive cells.

The combination of gefitinib with calcitriol or their synthetic analogs resulted in a greater antiproliferative effect than with either of the agents alone in EGFR and HER2 positive breast cancer

Abbreviations: CK-7, cytokeratin 7; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FSC-A, forward scatter area; FSC-H, forward scatter height; GI, growth inhibitory; HER2, epidermal growth factor receptor type II; IC, inhibitory concentration; MFI, mean fluorescence intensity; TKI, tyrosine kinase inhibitors; VDR, vitamin D receptor.

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cells. The mechanistic explanation for these results includes downregulation of MAPK signaling pathway, decrease of cells in G2/M phase and induction of apoptosis mediated by upregulation of BIM and activation of caspase 3.

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Contents

1. Introduction

The ErbB or epidermal growth factor receptor (EGFR) family consists of four members: EGFR/HER1/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. Ligand binding to the receptors induces the formation of homo- and heterodimers and activation of the kinase domain, resulting in phosphorylation of specific tyrosine residues. Phosphorylation triggers several signaling pathways such as the PI3K/Akt and the Ras/Raf/MEK/MAPK that promotes cell proliferation, survival, adhesion, migration and differentiation [1,2]. Although HER2 has no ligands, this receptor is activated via heterodimerization with ligand-activated ErbB receptors. HER2 overexpression occurs in about 20–30% of patients with breast cancer [3,4]. Notably, EGFR and HER3 are the major partners of HER2 [5,6]. Coexpression of EGFR/HER2 has been observed in 10–36% of all primary human breast carcinomas, and it has been associated with a more aggressive clinical behavior when compared with those tumors that express a single receptor [7–9]. Therefore, drugs that selectively inhibit these targets represent good therapeutic alternatives for HER2 and EGFR-positive breast cancer tumors. In this regard, small molecule tyrosine kinase inhibitors (TKIs) have been developed. Gefitinib, a TKI, has been approved by the FDA for treatment of advanced non-small cell lung carcinoma with activating EGFR mutations [10]. In addition, gefitinib has been shown to reduce cell proliferation and tumor growth in breast cancer cell lines or under *in vivo* conditions in xenografted animals with different levels of EGFR or HER2 expression [11–13]. Interestingly, gefitinib is more potent in inhibiting proliferation of breast cancer cells with a high and low levels of HER2 and EGFR respectively, compared to those cells with high levels of EGFR [11,13]. Gefitinib effects on HER2 and EGFR-expressing breast cancer cells are mediated by the inhibition of Akt and MAPK signaling pathways [11–14]. In addition, in these cells, gefitinib induces cell cycle arrest in G1 phase and an increase of pro-apoptotic BIM protein expression [2,11,15].

Calcitriol, through its nuclear vitamin D receptor (VDR), exerts an important antitumor activity [16]. In this regard, epidemiological studies have demonstrated an association between low levels of calcidiol, the precursor of calcitriol, with an increased risk of developing breast cancer and tumor progression [17,18]. VDR expression is found in 90% of all human breast tumors, which correlates with a longer disease-free survival compared with VDR-negative tumors [19]. Among the mechanism by which calcitriol exerts its antiproliferative activity are those related with cell cycle arrest, stimulation of cell differentiation and regulation of anti-apoptotic proteins [20–22]. Recent observations from this laboratory have shown the important antiproliferative effects of calcitriol alone or combined with other antineoplastic agents [23,24]. The main drawback for calcitriol clinical use is that high doses of calcitriol induces disturbances in calcium homeostasis [25]. To overcome this unwanted secondary effect of calcitriol, numerous synthetic vitamin D analogs have been developed. EB1089 and calcipotriol are vitamin D synthetic analogs that retain the ability to inhibit cell proliferation and induce cell differentiation while display reduced calcemic activity. Interestingly, calcitriol and its analog EB1089 downregulate EGFR levels in both ovarian and breast cancer cell lines [26,27].

Taking into account all these observations, the aim of this study was to investigate the effects of a combination of gefitinib with calcitriol or its analogs upon cell proliferation in breast cancer cells.

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