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Biflorin, A Naphthoquinone, Inhibitsegfr in Breast Cancer Cells

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Abstract

The ErbBreceptor family has been used as therapeutic target for treatment of several types of cancer. Biflorin is a natural *o*-naphthoquinone with anticancer properties. Herein, we described the effect of biflorin on cell growth and EGFR expression in *SK-Br3* human breast cancer cell line with high EGFR expression. Biflorin significantly inhibited breast cancer cell growth, in a dose- and time-dependent manner as determined by the Alamar Blue assay. Noncitotoxicity were observed in normal MCF-10A breast cell line. Furthermore, biflorin inhibited EGFR expression in a dose-dependent manner. Biflorin can be used as a drug lead for new molecules against EGFR.

Keywords: Biflorin; Breast cancer; EGFR inhibition

Introduction

Genes encoding receptor tyrosine kinases of the epidermal growth factor receptor (EGFR) family are commonly dysregulated in cancer. The members of the family regulate fundamental cell behavior including development, cell growth, survival, death, differentiation and proliferation [1-6]. Furthermore, EGFR playsa key role in metastasis, tumorigenesis, and drug resistance. Elevated aggressiveness associated with EGFR activity in cancer cells may be explained in part by the activation of Epithelial-Mesenchimal Transition (EMT) associated events [7]. More recently, studies have shown that microenvironment produce growth factors and they can convert differentiated cells to a more stem cell-like state [8], which are believed to be responsible for tumor initiation and maintenance. The EGF-EGFR signalling pathway could provide critical function for self-renewal of several tumours [9-11]. Overexpression of the EGFR receptors is associated with poor survival and ten-year survival is lower when Estrogen Receptor (ER) is positive in patients with breast cancer [12-14]. In this way, EGFR can be a good target to drug therapy, since the evolution of acquired resistance related to mutation within EGFR remains an obstacle to conventional and target therapy current on clinic [15,16].

Biflorin (Figure 1A) is a prenylated *ortho*-naphthoquinone (6,9-dimethyl-3-(4-methyl-3-pentenyl) naphtha-[1,8-bc]-pyran-7,8-dione), isolated from roots of *Caprariabiflora*L., a perennial shrub belonging to the family Schrophulariaceae. This species is widely distributed in the American continent, in countries like Peru, Guyana, Trinidad and Tobago, Mexico, Curacao, Bahamas and Guatemala. In Brazil, is distributed in the states of Minas Gerais, Goiás, Piauíand Espírito Santo [17].

The first phytochemical studies with *C.biflora*were developed by Gonçalves et al., who isolated from the roots of the plant, an active ingredient called biflorin, which showed bactericidal and fungicidal activity [18].

Biflorin have a similar structural a β-lapachone, (3,4-dihydro-2,2-dimethyl-2H-naphtol[1,2-b]pyran-5,6-dione)-1,2-napthoquinone [19]. β-lapachone have a strong cytotoxicity in tumor cells mainly melanoma, leukemia, colon, lung, breast and prostate and also shows to have a synergistic activity in radiotherapy of tumors. These effects can be attributed to induction of apoptosis and inhibition of complex DNA Topoisomerase, causing damage to DNA of cells cancer [20]. Biflorin has shown a strong cytotoxic activity against several tumor cell lines along with antitumor activity and survival increasing on mice bearing different kind of tumors indicating a promising antitumor use in clinics [21-23]. Furthermore, genotoxic, antimutagenic and protective effects of biflorin were recently demonstrated by Vasconcellos et al. [24]. In this way, breast cancer cells with high EGFR expression were treated with biflorin and growth inhibition and EGFR expression was evaluated.

Material and Methods

Cell culture and cell viability assays

SK-Br3 breast cancer cell and MCF10A normal breast cell were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured according to recommended specifications.

Alamar blue: *SK-Br3* cells seeded in 96-well plates (10^4 cells per well) were treated with biflorin (1, 2.5, 5, 10 and 20 μM) and the Alamar Blue™ assay was performed after 12, 24, 48, and 72 h [25].

Crystal violet stain: Cells (MCF-10A) seeded in 12-well plates (2×10^5 cells per well) were treated with biflorin 5, 10 and 20 μM for 24 h, fixed in 4% paraformaldehyde and stained with 0.1% crystal violet. Plates were scanned and the intensity of the stained wells was illustrated.

Western blot: *SK-Br3* cancer cells seeded in 6-well plates (2×10^5 cells per well) were treated with biflorin 5, 10 and 20 μM. After 24 h incubation western blot for EGFR was performed, according to manufacture.

Results

Cytotoxicity studies

Alamar Blue™ assay and crystal violet were used to access drug toxicity against *SK-Br3* human breast cancer cell line and MCF-10A normal breast cell line. Time- and concentration-dependent experiments were performed in order to elucidate drug selectivity and

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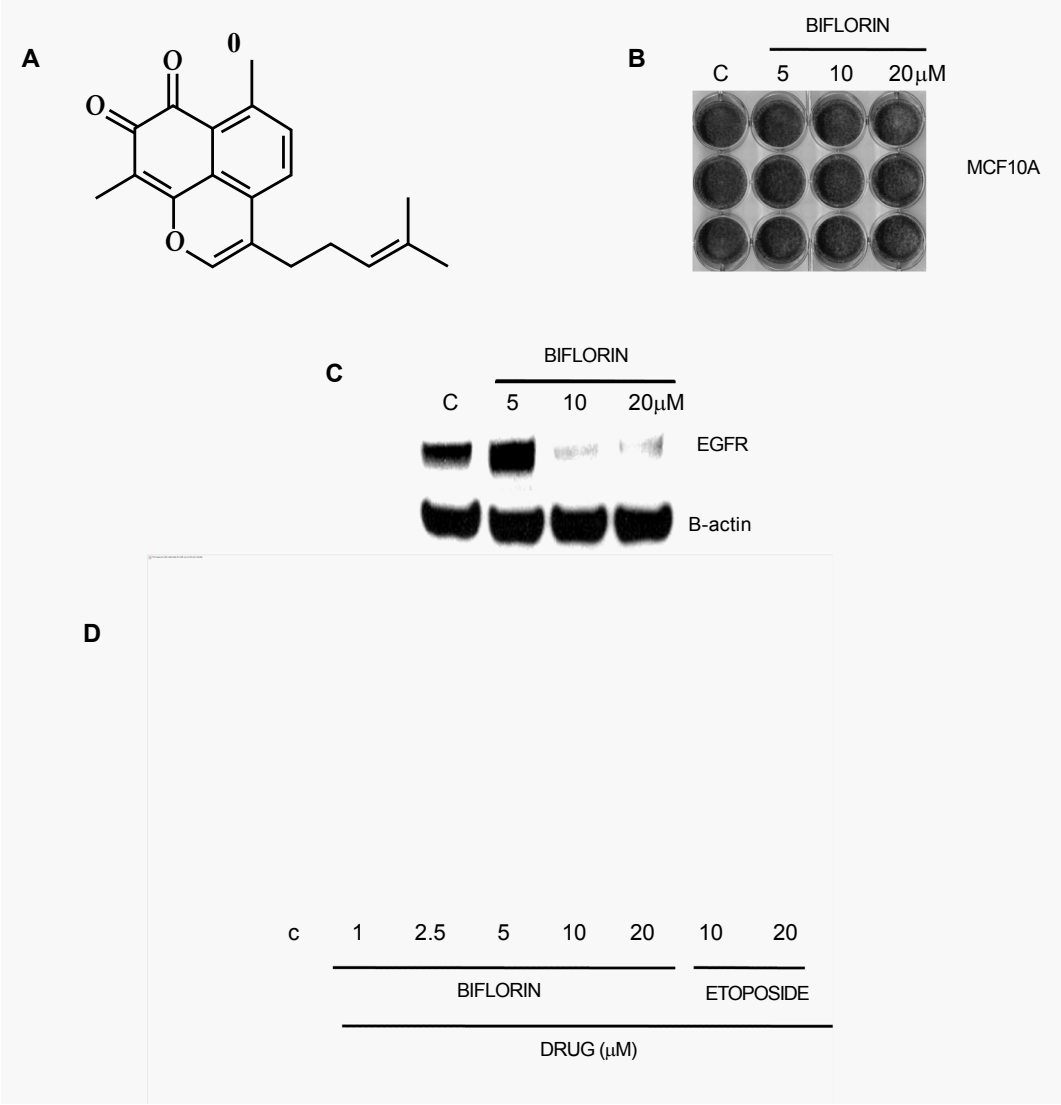

Figure 1: Biflorin inhibits cell growth by inhibiting EGFR expression. a) Biflorin chemical structure; b) Growth inhibition of biflorinon MCF-10A cells and stained with Crystal Violet; c) Effect of biflorin on expression of EGFR; d) Time- and Dose-dependent SK-BR3 growth inhibition. Etoposide was used as a positive control. The data shown on b, c and d are representative of results obtained in at least three separate experiments in triplicate.

the end point for expression experiments. Normal MCF-10A cells treated with biflorin did not displayed citotoxicity (Figure 1B). *SK-Br3* cancer cells treated with biflorin (1-10 μM) displayed citotoxicty at 24 h when compared to non-treated cells, however after 24 h they start to grow as the negative control. Growth inhibition was observed at 20 μM at all time-tested (Figure 1D). Interestingly, cells treated with etoposide (10 and 20 μM) recovered its growth at all tested concentration after 24 h, reaching its completely confluent stage after 48 h. These results suggest that cells resistant to etoposide may be susceptible to biflorin.

Expression study

Western Blot for EGFR was performed in order to determine its possible relationship with growth inhibition. *SK-Br3* cells treated with biflorin (10 and 20 μM) inhibit the EGFR expression (Figure 1C) leading to cell growth inhibition at 20 μM (Figure 1D).

Discussion

The role of growth factor and steroid hormone receptors in human breast cancer has been given considerable attention. Over expression of the EGFR and ErbB2 receptor tyrosine kinases is associated with poor survival in patients with breast cancer [12,13]. Ten-year survival is lower for patients with tumors positive for both Estrogen Receptor (ER) and ErbB1/ErbB2/ErbB3 than for those with ER+ tumors negative for all ErbB receptors [14]. Furthermore, growth factors like EGF has been related to resistance to endocrine agents in breast cancer, which makes these molecules a target for new drugs [26]. In this way, the purpose of this study was to evaluate the cytotoxicity of Biflorin in *SK-Br3* human breast cancer cells and its role in EGFR expression. *SK-Br3* highly expresses HER family members, EGFR, HER2 and HER3, making it a suitable model to study drugs that affect these receptors [27-29]. Single-agent therapy with direct targeted agents has shown limited success in tumor growth control, mainly because escape or resistance mechanisms [30]. Thus, there is a need for new drugs to target this receptor and control cell growth.

Bioactive phytochemicals compounds that are non-toxic at effective doses offer promising options for the development of effective

chemo- therapeutic or adjuvant therapy for conventional cytotoxic therapies. Biflorin is one of these compounds. Biflorin has a similar structure as β-lapachone, a natural 1,2-naphthoquinone with several activities against cancer *in vitro* and *in vivo* [31]. Their structures differ only on the side chain formed from the heterocyclic ring, where biflorin has three carbon unit longer than the β-lapachone making it more liposoluble. Furthermore, on the structure of biflorin the heterocyclic ring is linked to the 4,5 naphthoquinone carbon skeleton ring while the β-lapachone has in 3,4 carbon ring.

Previous studies by Vasconcellos et al. (2005; 2007; 2011) [21,22,24] demonstrated that biflorin have antitumor properties *in vitro* and *in vivo*. Moreover, the antimutagenic and protective effects of biflorin were recently demonstrated in *Salmonella thiphymurium,* in *Saccharomicescerevisiae*, and in V79 mammalian cells [21]. Our study demonstrates that treatment of *SK-Br3* cell line with biflorin displayed citotoxicity against cancer cell but not to normal cell. Furthermore, biflorin decreases the expression levels of total EGFR. There are few studies in the literature underlying the role of naphthoquinone in EGFR inhibition. Su et al. [32] showed that Furano-1,2-naphthoquinone inhibits A549 cancer cell growth and suppressed EGFR phosphorylation. More recently, Hafeez et al. [33] demonstrated that plumbagin inhibits the growth of pancreatic cancer cells both *in vitro* and *in vivo* and constitutive inhibited the expression of EGFR. Taking together, the results suggest that inhibition of cell proliferation by biflorin is mediated, at least in part, through the down-regulation of EGFR signaling pathway.

Several natural products has been reported to inhibit EGFR. Prasad and Katiyar [34] demonstrated that grape seed proanthocyanidins inhibits cell proliferation throughout EGFR inhibition. Also, Abbaoui et al. [35] described the inhibition of bladder cancer cell by broccoli sprout. The identification of new molecules is an important consideration in terms of cancer chemopreventive or cancer therapeutic strategies in suggesting potential combinations with other agents or drugs, and in elucidating the mechanisms of action of any test agent. In this context, the data present herein, suggests that biflorin can be used in tumors cells that show EGFR amplification and are resistant to etoposide. Moreover, biflorin can be a drug lead to new drugs that target EGFR. Further studies have to be performed to elucidate the mechanism underlying biflorincitotoxicity.

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