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A ferutinin analogue with enhanced potency and selectivity against ER-positive breast cancer cells *in vitro*

Rémi Safi[a,b], Aline Hamade[a], Najat Bteich[a], Jamal El Saghir[b], Mona Diab Assaf[c], Marwan El-Sabban[b,*,2], Fadia Najjar[a,*,1]

[a] Departments of Chemistry–Biochemistry and Biology, Laboratoire d'Innovation Thérapeutique, Faculty of Sciences II, Lebanese University, Lebanon
[b] Department of Anatomy, Cell Biology and Physiological Sciences, Faculty of Medicine, American University of Beirut, Beirut, Lebanon
[c] Department of Chemistry and Biochemistry, Faculty of Sciences II, Lebanese University, Lebanon

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ABSTRACT

Estrogen is considered a risk factor for breast cancer since it promotes breast-cell proliferation. The jaesckeanadiol-3-*p*-hydroxyphenylpropanoate, a hemi-synthetic analogue of the natural phytoestrogen ferutinin (jaesckeanadiol-*p*-hydroxybenzoate), is designed to be devoid of estrogenic activity. This analogue induces a cytotoxic effect 30 times higher than that of ferutinin towards MCF-7 breast cancer cell line. We compared these two compounds with respect to their effect on proliferation, cell cycle distribution and cancer stem-like cells in the MCF-7 cell line. Treatment with ferutinin (30 μM) and its analogue (1 μM) produced significant accumulation of cells at the pre G0/G1 cell cycle phase and triggered apoptosis. Importantly, this compound retains its anti-proliferative activity against breast cancer stem/progenitor cells that are naturally insensitive to ferutinin at the same dose. These results position ferutinin analogue as an effective compound inhibiting the proliferation of estrogen-dependent breast cancer cells and consistently targeting their stem-like cells.

1. Introduction

Breast cancer is the most common cancer and the second cause of cancer-related death among women [1]. Exposure to estrogen has been considered a risk factor for breast cancer because it promotes breast-cell proliferation [2]. The effects of 17β-estradiol (E2) and related compounds are mediated by two members of the nuclear receptor superfamily, the estrogen receptors (ER) α and β. Upon ligand binding, ERs undergo a conformational change allowing chromatin interaction and the transcriptional regulation of target genes [3]. ERα has proven to be a valuable predictive and prognostic factor in the clinical management of the disease. Approximately 70% of breast cancers are ERα-positive responsive to hormonal therapy. Therefore, inhibition of the ERα has become one of the major strategies for the prevention and treatment of breast cancer. This has further led to the discovery of effective anti-estrogens such as tamoxifen and ICI 182,780 and, in consequence, to the problem of anti-estrogen resistance, the mechanisms of which are not fully understood [4].

The emerging theory of drug resistance describes the existence of a highly tumorigenic subpopulation of breast cancer stem cells (BCSCs) within the tumor. Anti-cancer therapy that specifically targets BCSCs has yet to be demonstrated, mainly because differences in response to therapies between CSCs and normal tissue stem cells have not been clearly elucidated [5]. Additionally, many therapies currently in use are inadequate in terms of their therapeutic efficacy and of their undesirable side effects.

Ferula species are reputed in traditional medicine for their therapeutic applications against a range of disorders. They are a good source of phytochemical compounds such as terpenoid coumarins and sesquiterpene derivatives [6]. *Ferula* species have been shown to display growth-suppressive activity and chemopreventive properties without the adverse side effects normally associated with current chemotherapies [7,8]. Ferutinin is one of the most widely characterized phytoestrogen occurring in *Ferula* genus. It exhibits growth-suppressive

Abbreviations: 3D, three dimensions; BC, breast cancer; BCSCs, breast cancer stem cells; Ca^{2+}, calcium; CSCs, cancer stem cells; CTL, control; E2, 17β-estradiol; EDTA, ethylenediaminetetraacetic acid; ER, estrogen receptors; FBS, foetal bovine serum; FRT, ferutinin; HAEC, human aortic endothelial cells; HRT, hormonal replacement therapies; IC_{50}, half-maximum inhibitory concentration; p-ER, phosphorylated form of ER; PI, propidium iodide; SC, stem cell; SD, standard deviation; SERM, selective estrogen receptor modulator; SFU, sphere-forming unit

* Corresponding authors.
E-mail addresses: me00@aub.edu.lb (M. El-Sabban), fnajjar@ul.edu.lb, fadia.najjar@gmail.com (F. Najjar).
[1] Present address :Lebanese University - Sciences Faculty section II, Department of Chemistry and Biochemistry, P.O box: 90656, Jdeidet el Metn – Lebanon.
[2] Present address :American University of Beirut Medical Center. Bldg.: Diana Tamari Sabbagh Bldg, DTS-316 Street Bliss – Lebanon.

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potential in a wide variety of tumor cells [9–15] and in animal cancer models without any noticeable systemic toxicity[16]. Although the anti-proliferative activity of ferutinin is well documented, its effect on CSC is not yet investigated. Ferutinin is considered a selective estrogen receptor modulator (SERM) acting as an agonist to ERα (IC_{50} = 33.1 nM) and an agonist/antagonist to ERβ (IC_{50} = 180.5 nM) [17,18]. Recently, much attention has been given to its estrogenic activity when developing new hormonal replacement therapies (HRT) for osteoporosis patients [19]. Unlike estrogens currently employed in HRT, ferutinin treatment provides a protective function against uterine carcinoma [20–23]. Despite its remarkable estrogenic properties, ferutinin was documented for its anti-cancer activity in estrogen-dependent breast cancer cells. Reported data on the effect of ferutinin on the proliferation of estrogen-dependent cells is controversial. While Lhuillier et al. showed that ferutinin exerts a proliferative effect at low concentrations and an anti-proliferative effect at higher concentrations [11].

We have previously demonstrated that high concentration of ferutinin inhibited the proliferation of both estrogen-dependent (MCF-7) and -independent (MDA-MB-231) breast cancer cells. These findings prompted us to enhance the anti-proliferative activity of this parent molecule by synthesizing novel analogues *via* chemical modification of ferutinin. Among these analogues, the jaesckeanadiol-3-p-hydroxyphenylpropanoate, having an extension of two carbons with an unsaturated bond (Fig. 1), showed a potent cytotoxicity and higher selectivity towards MCF-7 cells [24]. Interestingly, MCF-7 cells express substantial levels of ER which represent a large sub-group of estrogen-responsive breast cancers and mimic the majority of invasive human breast cancers that express ER. Considering the high prevalence of estrogen-positive breast carcinoma, MCF-7 cell line constitutes a useful *in vitro* tool for potential drug screening.

In the present work, we addressed a comparative study between ferutinin and its potent analogue on diverse cellular events, such as cell viability, cell cycle distribution, apoptosis and stem-like cell enrichment of estrogen-dependent (MCF-7) breast cancer cell line.

2. Material and methods

2.1. Cell lines and reagents

MCF-7 cell line was purchased from the American Type Culture Collection (ATCC, USA) and cultured in RPMI-1640 (Lonza, Switzerland) containing 10% fetal bovine serum (FBS) (Sigma, St Louis, MO, USA) and 1% v/v penicillin-streptomycin (Sigma). Cells were grown to confluence at 37 °C in a humidified atmosphere containing 5% CO_2 in air and were passaged weekly using 1% trypsin (Gibco, USA) to maintain the optimum conditions for exponential growth. Ferutinin and its analogue were hemi-synthetized as described earlier [24].

2.2. MTT cell proliferation assay

MCF-7 cells were cultured in 96-well plates with a density of 3000 cells/well. After 24 h, exponentially growing cells were exposed to ferutinin and its analogue, in triplicate, at final concentrations ranging from 0.1 to 60 µM. After 24, 48 and 72 h of incubation, 5 mg/ml of MTT reagent (Promega, USA) was added and cultured for 4 h, and then solubilization solution was added (100 µl/well). The optical density is measured, after 24 h of incubation, at 570 nm by the Multiskan Ex spectrum. Results are presented as the average of three wells. The percent of cells surviving was determined by comparing the average absorbance with the standard deviation (SD) of three wells per concentration of the treated cells with the average absorbance SD of three wells per concentration of the non-treated cells. Results are presented as percent of control. The final results were obtained in one independent experiment run in triplicate.

2.3. Cell cycle analysis

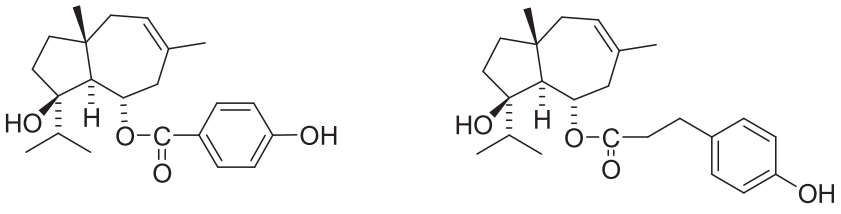
Ferutinin- and analogue-treated cells were washed twice with cold PBS, fixed in ice cold ethanol, and stored for 24 h at −20 °C. Subsequently, cells were rinsed with PBS and stained with propidium iodide (PI) (1 mg/ml) (Sigma). Cell cycle analysis was performed using a flow cytometer, BD FACSDiva (BD Biosiences, CA, USA). Each sample was collected as 10 000 ungated events and analyzed using Cell-Quest software. Results are representative of two independent experiments.

2.4. Hoechst 33342 staining of apoptotic cells

To examine the apoptotic changes in MCF-7 cells, Hoechst 33342 (Molecular probes, Oregan, USA) nuclear staining assay was performed. For monolayer cultures 25,000 cells/well were seeded in 12 well plates. After 40–70% confluency, the cells were treated with different concentrations of ferutinin (30 µM) or ferutinin's analogue (1 µM) calculated from the IC_{50} values, for 72 h. After completion of treatment the cells were fixed with methanol for 30 min at 4 °C in the dark. Fixed cells were washed twice with PBS, and then Hoechst 33342 solution was spread over the plates followed by incubation for 10 min in the dark. Labeled cells were washed repeatedly with PBS to remove the excess of Hoechst 33342 stain and evaluated under a fluorescence microscope (Zeiss Z.1, Germany).

2.5. Sphere formation assay and sphere passage

Spheres were collected after centrifugation, then dissociated with trypsin-EDTA and mechanically disrupted with a pipette. The resulting single cells were then centrifuged to remove the enzyme and re-suspended in low serum medium (2%) allowed to form spheres. The spheres were passaged every 12 days. The dissociated single sphere-forming cells were also diluted to a density of 1000 cells/well. Then, the 25 µl/well diluted cell suspension in low serum medium was plated into 24-well plate, and 25 µl of Matrigel™ (BD Biosiences) was added. The wells with only one cell were marked and observed every day. For propagations, cells were detached from the Matrigel™ by the addition of dispase enzyme for 1 h, then dissociated with trypsin-EDTA and sheared with a syringe. The single cells resulting were centrifuged, re-suspended into low serum media, and then plated with the same procedure. At every generation, cells were divided into control non-treated pool and treated pool as shown in the experimental diagram. Ferutinin (Fig. 2A)



a b

Fig. 1. Structure of ferutinin (a) and its analogue (b).