Molecular and Cellular Endocrinology xxx (2017) 1–9

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

A functional drug re-purposing screening identifies carfilzomib as a drug preventing 17β-estradiol: ERα signaling and cell proliferation in breast cancer cells

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ARTICLE INFO

Article history:
Received 17 May 2017
Received in revised form
27 July 2017
Accepted 27 July 2017
Available online xxx

Keywords:
17β-estradiol
Estrogen receptor
Breast cancer
Drug screening
Carfilzomib

ABSTRACT

Most cases of breast cancer (BC) are estrogen receptor α-positive (ERα+) at diagnosis. The presence of ERα drives the therapeutic approach for this disease, which often consists of endocrine therapy (ET). 4OH-Tamoxifen and faslodex (*i.e.,* fulvestrant - ICI182,780) are two ETs that render tumor cells insensitive to 17β-estradiol (E2)-dependent proliferative stimuli and prevent BC progression. However, ET has limitations and serious failures in different tissues and organs. Thus, there is an urgent need to identify novel drugs to fight BC in the clinic. Re-positioning of old drugs for new clinical purposes is an attractive alternative for drug discovery. For this analysis, we focused on the modulation of intracellular ERα levels in BC cells as target for the screening of about 900 Food and Drug Administration (FDA) approved compounds that would hinder E2:ERα signaling and inhibit BC cell proliferation. We found that carfilzomib induces ERα degradation and prevents E2 signaling and cell proliferation in two ERα+ BC cell lines. Remarkably, the analysis of carfilzomib effects on a cell model system with an acquired resistance to 4OH-tamoxifen revealed that this drug has an antiproliferative effect superior to faslodex in BC cells. Therefore, our results identify carfilzomib as a drug preventing E2:ERα signaling and cell proliferation in BC cells and suggest its possible re-position for the treatment of ERα+ BC as well as for those diseases that have acquired resistance to 4OH-tamoxifen.

1. Introduction

17β-estradiol (E2) regulates the physiology of reproductive and non-reproductive organs both in males and in females by binding to the estrogen receptors (*i.e.,* ERα and ERβ) and thus it is a general regulator of body homeostasis (Acconcia et al., 2016). The E2 pleiotropic nature resides in its ability to elicit diverse intracellular effects (including cell proliferation) through multifaceted signaling mechanisms. As nuclear receptors, ERα and ERβ localize into the nucleus where they mediate changes in gene transcription profiles in response to E2 (*i.e.,* nuclear effects). ERα and ERβ also localize at the cell plasma membrane where they activate E2 extra-nuclear signals. Nuclear and extra-nuclear events cross-talk and are both required for cell and organ physiology (Acconcia et al., 2016; Ascenzi et al., 2006; Levin and Hammes, 2016). Many E2 physiological effects occur while E2 finely tunes ERα and ERβ intracellular levels. This modulation is specific for each receptor subtype with E2 reducing ERα intracellular levels and inducing ERβ intracellular accumulation. Therefore, E2 signaling and its resulting physiological functions further depend on the dynamic temporal variations in ERs cell content (Acconcia et al., 2016; Leclercq et al., 2006; Thomas and Gustafsson, 2011). In turn, deregulation of both E2 intracellular signaling and control of ERs intracellular levels contributes to the development of diverse endocrine-related diseases including breast cancer (BC) (Acconcia et al., 2016; Ascenzi et al., 2006; Levin and Hammes, 2016; Leclercq et al., 2006; Thomas and Gustafsson, 2011).

In 70% of all cases, BC growth depends on E2 signaling through ERα (*i.e.,* ERα+ tumors) as E2 is a mitogen for BC cells, works as a survival and anti-apoptotic factor and induces cell invasion and migration (Acconcia and Marino, 2011). The clinical approach for ERα+ tumors targets different aspects of oncogenic E2:ERα signaling: aromatase inhibitors (AIs) are used to inhibit E2

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http://dx.doi.org/10.1016/j.mce.2017.07.027
0303-7207/

synthesis thus reducing systemic E2 availability; 4OH-tamoxifen (*i.e.,* the actual mainstay for treatment of ERα+ tumors) is the prototype of the selective ER modulators (SERMs), it binds ERα and inhibits receptor to work as a transcription factor, thus blocking E2-dependent gene expression; and faslodex (*i.e.,* fulvestrant or ICI182,780) is the prototype of the selective ER down-regulators (SERDs), physically engages with ERα and induces its 26S proteasome-dependent degradation (Ferlay et al., 2013; Lumachi et al., 2011), thus eliminating ERα from BC cells.

However, the use of such drugs has limitations: AIs are typically reserved for postmenopausal women with BC and produce musculoskeletal failures, and 4OH-tamoxifen and faslodex possess serious side effects (*e.g.,* endometrial cancer for 4OH-tamoxifen) and often determine the development of BC cells resistant to such anti-cancer ET (Angus et al., 2017; Tryfonidis et al., 2016). Consequently, considerable drug discovery research efforts aimed to identify novel SERMs and SERDs but 4OH-tamoxifen and faslodex stand still as the unique ERα ligands available for BC treatment (Ferlay et al., 2013; Lumachi et al., 2011). Thus, additional drugs to treat BC are required. Novel compounds that inhibit ERα+ BC progression can be identified either by searching for natural or synthetic compounds that bind to and/or inhibit ERα or by defining and targeting a critical parameter required for E2:ERα signaling-induced cell proliferation.

We previously hypothesized that such parameter can be the modulation of ERα intracellular levels (Acconcia et al., 2016; Totta et al., 2016b) as i) it is known since many years that both artificial ERα down-modulation in ERα+ cell lines and its over-expression in ERα-negative cells cause E2 insensitivity or cell death (Oesterreich et al., 2001; Jiang and Jordan, 1992); ii) ERα ligands influence receptor intracellular concentration: E2 reduces ERα intracellular levels and fuels cell proliferation (Leclercq et al., 2006; La Rosa et al., 2012; Totta et al., 2014), 4OH-tamoxifen and faslodex alter ERα content, with the former stabilizing it, the latter de-stabilizing it and either drugs blocking E2-induced BC cell proliferation (Leclercq et al., 2006); iii) molecules (*e.g.,* chloroquine) that do not bind to ERα influence ERα levels by affecting specific cellular pathways and prevent E2-induced BC cell proliferation (Totta et al., 2014; Cook et al., 2014); and iv) reduction in the intracellular content of proteins with functions unrelated to E2 signaling (*e.g.,* endocytic proteins) can change ERα content and inhibit E2-induced BC cell proliferation (Totta et al., 2015, 2016a). In turn, all this evidence suggests that molecules that directly or indirectly deregulate the control mechanisms for ERα intracellular abundance could have in principle the potential to inhibit E2-dependent proliferation in BC cells.

Herein, we challenged this hypothesis by screening a compound library with the aim to identify molecules that modify ERα intracellular levels. We report the discovery of carfilzomib (Kyprolis®) as an ERα-degrading drug that prevents the basal and E2-induced BC cell proliferation.

2. Materials and methods

2.1. Cell culture and reagents

17β-estradiol, DMEM (with and without phenol red) and fetal calf serum were purchased from Sigma-Aldrich (St. Louis, MO). Bradford protein assay kit as well as anti-mouse and anti-rabbit secondary antibodies were obtained from Bio-Rad (Hercules, CA). Antibodies against ERα (F-10 mouse), cyclin D1 (H-295 rabbit), cathepsin D (H75 rabbit), pS2 (FL-84 rabbit), p53 (DO-1 mouse) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-vinculin antibody was from Sigma-Aldrich (St. Louis, MO). MG-132 was purchased by Calbiochem (San Diego, CA). Chemiluminescence reagent for Western blotting was obtained from BioRad Laboratories (Hercules, CA, USA). Faslodex (*i.e.,* fulvestrant or ICI182,780) and 4OH-tamoxifen were purchased by Tocris (USA). FDA-approved drug library was purchased by Selleck Chemicals (USA). All the other products were from Sigma-Aldrich. Analytical- or reagent-grade products were used without further purification. The identities of all of the used cell lines [*i.e.,* human breast carcinoma cells (MCF-7; ZR-75-1)] were verified by STR analysis (BMR Genomics, Italy).

2.2. Cellular and biochemical assays

Cells were grown in 1% charcoal-stripped fetal calf serum medium for 24 h and then stimulated with E2 at the indicated time points. Where indicated, cells were treated with E2 (1 nM), carfilzomib (100 nM) or Faslodex (*i.e.,* fulvestrant or ICI182,780) (ICI) (100 nM). Protein extraction and biochemical assays were performed as described previously (Totta et al., 2016b). For cell number analysis, the CyQUANT® Cell Proliferation Assay (Life Technologies) was used according to the manufacturer's instructions. Specifically, MCF-7, ZR-75-1 and 4OH-Tamoxifen resistant MCF-7 cells (TamRes) were treated as described below. After counting 3000 cells were plated in 96-well plates in triplicate. After 4 h, the CyQUANT® Assay was performed at time 0 (*i.e.,* plated cells). In other 96-well plates, drugs were administered to cells in 1% charcoal-stripped fetal calf serum medium, and the CyQUANT® assay was performed after 48 h. For growth curves analysis, parental and TamRes MCF-7 cells were plated in growing medium and counted at the indicated time points after drug administration. Western blotting analyses were performed by loading 20–30 μg of protein on SDS-gels. Gels were run and transferred to nitrocellulose membranes with Biorad Turbo-Blot semidry transfer apparatus. Immunoblotting was carried out by incubating membranes with 5% milk (60 min), followed by incubation o.n. with the indicated antibodies. Secondary antibody incubation was continued for an additional 60 min. Bands were detected using a Biorad Chemidoc apparatus.

2.3. Cell cycle analysis

After treatments, cells were grown in 1% FBS for 24 h, harvested with trypsin, and counted to obtain 10^6 cells per condition. Then, the cells were centrifuged at 1500 rpm for 5 min at 4 °C, fixed with 1 ml ice-cold 70% ethanol and subsequently stained with PI buffer (500 μg/ml Propidium Iodide, 320 μg/ml RNaseA, in 0.1% Triton X in PBS). DNA fluorescence was measured using a CytoFlex flow cytometer and the cell cycle analysis was performed by CytExpert v1.2 software (Beckman Coulter).

2.4. Statistical analysis

A statistical analysis was performed using the ANOVA (One-way analysis of variance and Tukey's as post-test) test with the InStat version 3 software system (GraphPad Software Inc., San Diego, CA). Densitometric analyses were performed using the freeware software Image J by quantifying the band intensity of the protein of interest respect to the relative loading control band (*i.e.,* vinculin or tubulin) intensity. In all analyses, p values < 0.01 were considered significant, except of the densitometric analyses with a choosen threshold of $p < 0.05$. Z' factor and robust Z scores calculation was performed according to (Zhang et al., 1999) and (Zhang, 2011), respectively.

Please cite this article in press as: Busonero, C., et al., A functional drug re-purposing screening identifies carfilzomib as a drug preventing 17β-estradiol: ERα signaling and cell proliferation in breast cancer cells, Molecular and Cellular Endocrinology (2017), http://dx.doi.org/10.1016/j.mce.2017.07.027