Journal of Steroid Biochemistry & Molecular Biology 152 (2015) 76–83

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

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

Wedelolactone induces growth of breast cancer cells by stimulation of estrogen receptor signalling

Tereza Nehybova [a], Jan Smarda [a,b], Lukas Daniel [c,d], Jan Brezovsky [c,d], Petr Benes [a,d,*]

[a] Laboratory of Cellular Differentiation, Department of Experimental Biology, Faculty of Science, Masaryk University, Kamenice 5/A36, 625 00 Brno, Czech Republic
[b] Masaryk Memorial Cancer Institute, RECAMO, Zluty kopec 7, 656 53 Brno, Czech Republic
[c] Loschmidt Laboratories, Department of Experimental Biology and Research Centre for Toxic Compounds in the Environment RECETOX, Faculty of Science, Masaryk University, Kamenice 5/A13, 625 00 Brno, Czech Republic
[d] International Clinical Research Center, Center for Biological and Cellular Engineering, St. Anne's University Hospital, Pekarska 53, 656 91 Brno, Czech Republic

ARTICLE INFO

Article history:
Received 17 December 2014
Received in revised form 9 April 2015
Accepted 26 April 2015
Available online 28 April 2015

Keywords:
Breast cancer
Estrogen receptor
Phytoestrogen
Wedelolactone

ABSTRACT

Wedelolactone, a plant coumestan, was shown to act as anti-cancer agent for breast and prostate carcinomas *in vitro* and *in vivo* targeting multiple cellular proteins including androgen receptors, 5-lipoxygenase and topoisomerase IIα. It is cytotoxic to breast, prostate, pituitary and myeloma cancer cell lines *in vitro* at μM concentrations. In this study, however, a novel biological activity of nM dose of wedelolactone was demonstrated. Wedelolactone acts as agonist of estrogen receptors (ER) α and β as demonstrated by transactivation of estrogen response element (ERE) in cells transiently expressing either ERα or ERβ and by molecular docking of this coumestan into ligand binding pocket of both ERα and ERβ. In breast cancer cells, wedelolactone stimulates growth of estrogen receptor-positive cells, expression of estrogen-responsive genes and activates rapid non-genomic estrogen signalling. All these effects can be inhibited by pretreatment with pure ER antagonist ICI 182,780 and they are not observed in ER-negative breast cancer cells. We conclude that wedelolactone acts as phytoestrogen in breast cancer cells by stimulating ER genomic and non-genomic signalling pathways.

1. Introduction

Steroidal estrogens are important signalling molecules that act *via* genomic and non-genomic transduction mechanisms. Phytoestrogens are plant-derived compounds that structurally and/or functionally mimic mammalian estrogens by direct interaction with nuclear and extranuclear estrogen receptors (ERs) [1]. Phytoestrogens belong to diverse classes of compounds and are believed to provide benefits to human health although this is still matter of intense debate [2].

Wedelolactone is naturally occurring coumestan isolated from *Eclipta alba* and *Wedelia calendulacea* [3]. Traditional Asian and South American medicine uses these plants to treat hepatitis, snake venom poisoning and viral infection [3,4]. Compounds extracted from *Wedelia chinensis* synergistically suppress growth of prostate cancer cell lines both *in vitro* and *in vivo* [5,6]. Furthermore, the growth inhibitory and proapoptotic effects of wedelolactone itself were documented in various cancer cell lines including breast, colon, hepatocellular, pituitary and neuroblastoma [7–11]. The anti-cancer effects of wedelolactone have been attributed to the inhibition of various protein kinases (IKK, FAK, ERK, PKC), androgen receptor, DNA topoisomerase IIα, and 5-lipoxygenase [5–13].

Wedelolactone is structurally related to another natural coumestan, a coumestrol (Fig. 1). Coumestrol is a phytoestrogen acting as agonist of both ERα and ERβ [14,15]. Although wedelolactone was suggested to act as phytoestrogen [16,17], any direct evidence for this statement has not been published yet. Interestingly, Lim et al. reported the absence of the luciferase reporter gene expression from

Abbreviations: AP-1, activator protein 1; CHFCS, charcoal stripped fetal bovine serum; CCND1, cyclin D1; CTSD, cathepsin D; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; ER, estrogen receptor; ERE, estrogen-responsive element; ERK, extracellular receptor kinase; FCS, fetal calf serum; HEK-293, human embryonic kidney 293 cell line; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; PDB, protein data bank; PBS, phosphate buffered saline; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; RLU, relative light units; RPMI, Roswell Park Memorial Institute medium; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

* Corresponding author at: Department of Experimental Biology, Faculty of Science, Masaryk University, Kotlarska 2, 611 37 Brno, Czech Republic.
Tel.: +420 54949 3125; fax: +420 54949 5533.
E-mail address: pbenes@sci.muni.cz (P. Benes).

http://dx.doi.org/10.1016/j.jsbmb.2015.04.019
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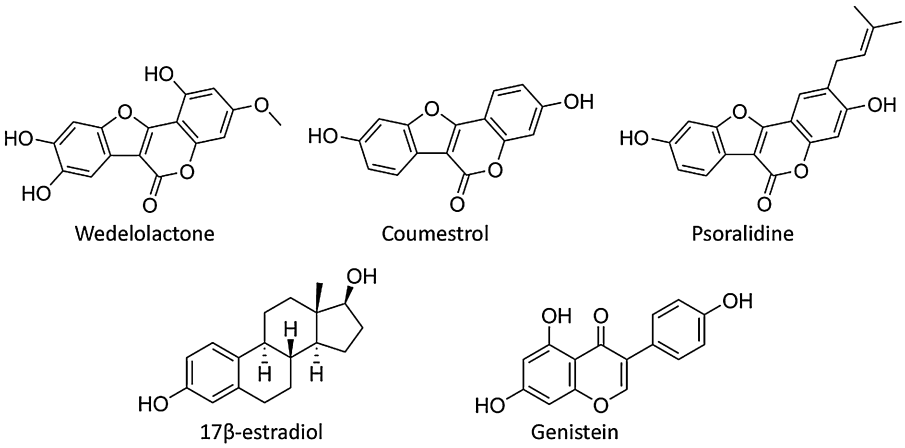

Fig. 1. Chemical structures of coumestans, 17β-estradiol and genistein.

ERE in wedelolactone-treated breast cancer MCF-7 cells [18]. Moreover, Xu et al. recently reported anti-estrogenic properties of 3-butoxy-1,8,9-trihydroxy-6H-benzofuro[3,2-c]benzopyran-6-one, the new wedelolactone derivative [19]. Therefore, the aim of this study is to analyze the estrogenic properties of wedelolactone in breast cancer cells in detail. We show that wedelolactone acts as phytoestrogen indeed by stimulating genomic and non-genomic signalling from estrogen receptors.

2. Material and methods

2.1. Chemicals and plasmids

Wedelolactone, 17β-estradiol, and ICI 182,780 were all purchased from Sigma–Aldrich (Sigma, St. Louis, MI). The ERE-luc plasmid was obtained from Panomics, Inc. (Panomics, Inc., Fremont, CA); pCDNA3.1-nv5-ERβ, pEGFP-C1-ERα described by Stenoien et al. and Wittmann et al. were obtained from Addgene (Addgene, USA) [20,21], CMV-βgal and p3TP-lux were described previously [22].

2.2. Cell culture

The human breast cancer cell lines MDA-MB-231 (often referred as ER-negative but expressing low amount of ERβ), MCF-7 and T47D (both expressing ERα and ERβ) were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. Human embryonic kidney 293 cell line (HEK-293) was grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Both wedelolactone and 17β-estradiol were used in 10 nM concentrations. To inhibit ER activation, cells were pretreated with 100 nM ICI 182,780 for 4 h.

2.3. Cell proliferation assay

To test proliferation rates, 1×10^5 MDA-MB-231, MCF-7 and T47D cells were seeded in 2 ml of growth medium in 6-well plates. Next day, the cells were washed with PBS and growth medium was replaced with RPMI 1640 lacking phenol red supplemented with 2% charcoal stripped fetal bovine serum (CHFCS), 2 mM L-glutamin, 100 U/ml penicillin, and 100 μg/ml streptomycin (steroid depleted medium). Next day, the cells were pretreated with either antiestrogen ICI 182,780 or solvent (DMSO) in fresh steroid depleted medium. Then, the cells were exposed to wedelolactone, 17β-estradiol or DMSO as a solvent for three days. Cells were counted daily using Casy®RT cell counter (Roche-Innovatis, Basel, Switzerland). To determine EC50 values, MCF-7 and T47D cells were exposed to various concentrations of wedelolactone for three days and counted. EC50 values were calculated using GraphPad PRISM 6 software (GraphPad-San Diego, CA, USA).

2.4. Determination of ER and AP-1 activities by the luciferase reporter assay

To test transactivation rates of ER and AP-1, 5×10^5 cells were seeded in 2 ml of regular growth medium in 6-well plates and cultured for 24 h. Transfection was performed using 8 μl of the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) with a mixture containing 1 μg of reporter plasmid (ERE-luc or p3TP-lux) and 1 μg of CMVβgal reference plasmid. Six hours later, the medium was replaced with the steroid-depleted medium with/without 100 nM ICI 182,780 for 4 h. Then, 10 nM wedelolactone, 10 nM 17β-estradiol or DMSO were added. Cells were incubated for 24 h, harvested and processed for luciferase and β-galactosidase assays as described elsewhere [22]. The luciferase activity of each sample was normalized for transfection efficiency according to the β-galactosidase activity.

2.5. Immunoblotting

Cells were seeded at the density 5×10^5 cells/well in growth medium in 6-well plates. Next day, the cells were washed with PBS and exposed to 100 nM ICI 182,780 or solvent in steroid depleted medium for 4 h. Then, the cells were treated with 10 nM wedelolactone, 10 nM 17β-estradiol or DMSO for 30 min (analysis of rapid non-genomic ER signalling) or 24 h (analysis of genomic ER signalling). Cells were harvested and lysed by boiling in a buffer containing 0.1 M Tris (pH 6.8), 16% v/v glycerol, 3.2% w/v SDS, 10% v/v β-mercaptoethanol, and 0.005% w/v bromophenol blue for 5 min. Cell lysates were subjected to SDS–PAGE and immunoblotted. Sample loading was normalized according to the protein concentration determined by DC protein assay (Biorad, Hercules, CA). Blots were probed with anti-p-c-Jun (Ser 73), anti-c-Jun (60A8, Cell Signalling Technology, Inc., Beverly, MA), anti-cyclin D1, anti-c-Myc, anti-ERK 1 (sc-246, sc-42 and sc-93, Santa Cruz Biotechnology Inc., Heidelberg, Germany), anti-cathepsin D (610801, BD Transduction Laboratories, San José, CA), or anti-p-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (4370, Cell Signalling Technology, Inc., Beverly, MA) antibodies. To control for sample loading, blots were probed with a α-tubulin specific antibody (T9026, Sigma, St. Louis, MI). The blots were then probed with secondary antibodies conjugated with peroxidase (Sigma, St. Louis,