Journal of Steroid Biochemistry & Molecular Biology xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

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

The anti-proliferative effect of 2-[piperidinoethoxyphenyl]-3-[4-hydroxyphenyl]-2H-benzo(b) pyran is potentiated via induction of estrogen receptor beta and p21 in human endometrial adenocarcinoma cells

Q1 I. Fatima[a], R. Saxena[a], G. Kharkwal[a], M.K. Hussain[b], N. Yadav[b], K. Hajela[b], P.L. Sankhwar[c], A. Dwivedi[a,*]

[a] Division of Endocrinology, CSIR-Central Drug Research Institute, Lucknow 226001, U.P., India
[b] Division of Medicinal and Process Chemistry, CSIR-Central Drug Research Institute, Lucknow 226001, U.P., India
[c] Department of Obstetrics & Gynecology, King George's Medical University, Lucknow 226001, U.P., India

ARTICLE INFO

Article history:
Received 24 August 2012
Received in revised form 15 April 2013
Accepted 16 April 2013

Keywords:
Endometrial cancer
Estrogen receptor
Transcriptional activation
Benzopyran derivative

ABSTRACT

In an effort to develop novel therapeutic agents for endometrial cancer, benzopyran derivatives synthesized at our institute display significant inhibitory activity on cellular growth in uterine cancer cells. The current study was undertaken to demonstrate and explore the estrogen receptor (ER) subtype mediated mechanism of action of benzopyran derivative 2-[piperidinoethoxyphenyl]-3-[4-hydroxyphenyl]-2H-benzo(b) pyran (K-1) in human endometrial cancer cells. K-1 competitively inhibited the estradiol binding to human ERα and ERβ and showed growth inhibitory activity in human endometrial Ishikawa, HEC1B and primary endometrial adenocarcinoma cells. Transient transactivation assays carried out in COS-1 cells have demonstrated the diminished ERα-ERE mediated- and induced the ERβ-ERE mediated-transactivation triggered by compound. It also induced ER-mediated transactivation of the cyclin-dependent kinase inhibitor (CDKI) $p21^{WAF-1}$ in both COS-1 cells and in Ishikawa cells. ERβ inducing effects of compound were blocked by ICI182,780. In endometrial adenocarcinoma cells, it induced ERβ and p21 expression significantly whereas the expression of fos, jun and ERα were significantly reduced. In addition, compound promoted ERα–β heterodimerization as observed in Ishikawa cells. These results demonstrate that the benzopyran compound suppressed the cellular growth via ERβ agonism, induction of p21 and via promoting the ERα–β heterodimerization, in addition to its antagonistic effects exerted on ERα, in human endometrial cancer cells. The study suggests that the dual action of benzopyran molecule may be of significant therapeutic value in ERα/β-positive cases of endometrial cancer.

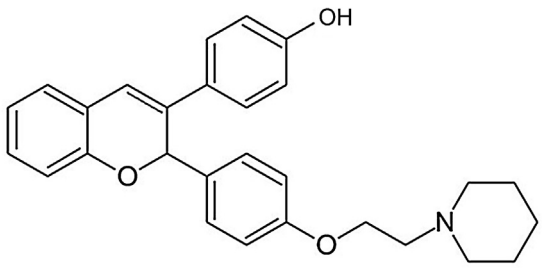
1. Introduction

Endometrial cancer has been increasing in frequency due to an aging population and changes in dietary and hormonal factors [1–3]. It is well documented that the mitogenic action of estrogens is critical in the etiology and progression of human breast cancer, endometrial cancer and other gynecological cancers [4,5]. Type 1 or those tumors of endometrioid histology, comprise 80% of cases and are thought to arise from persistent unopposed estrogen stimulation. The proliferative action of estrogen receptor-α (ERα) largely accounts for the carcinogenic activity of estrogens [6]. By contrast, earlier studies showed that estrogen receptor-β (ERβ) displays tumor-suppressor properties, thus supporting the interest to identify compounds which could increase its activity [7,8]. This may be due to their complex mechanism of action, which implies the binding to either ERα or ERβ, the recruitment of specific coregulators, formation of heterodimers, and the activation of target gene transcription [9–11]. A growing body of evidence has defined the protective role played by ERβ against uncontrolled cell proliferation in various types of cancers. Studies have shown that the expression of ERβ in the lesions of atypical hyperplasia and endometrial carcinoma was dramatically decreased as compared to that in the adjacent normal lesions [12]. The possibility of the induction of ERβ-mediated inhibition of cell proliferation by ERβ-selective ligands might therefore be an important research area for development of novel thearapeutic agents against endometrial cancer.

Different classes of synthetic compounds have been developed so far which are capable of antagonizing ERα action in reproductive tissues and, in particular, of blocking estradiol-stimulation of cellular growth in uterine tissues [13]. Among these,

* Corresponding author. Tel.: +91 0522 2612411–18x4385; fax: +91 0522 2623405/2623938.
E-mail address: anila.dwivedi@rediffmail.com (A. Dwivedi).

0960-0760/$ – see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.jsbmb.2013.04.005



2-[piperidinoethoxyphenyl]-3-[4-hydroxy phenyl]-2H-benzo(b)pyran (K-1)

Fig. 1. Chemical structure of 2-[piperidinoethoxyphenyl]-3-[4-hydroxyphenyl]-2H-benzo(b) pyran (K-1).

benzopyrans have been recognized as potent anti-estrogens and have high oral bio-availability [14,15]. These compounds show high affinity for estrogen receptor in human breast cancer and normal uterine cytosol and have estrogen antagonistic activity in human breast cancer models studied in vitro and in vivo in nude mice [16–18]. In a quest to design nonsteroidal pure antiestrogens, benzopyran derivatives synthesized at Central Drug Research Institute, India, displayed significant anti-estrogenic activity and inhibit uterine growth [19–21]. Our recent studies have shown the anti-proliferative activity of such three benzopyran derivatives in endometrial carcinoma cells [22] and it was reported that 2,3-diaryl-2H-1-benzopyran derivatives induce apoptosis via interfering with classical and non-classical ER signaling pathways and inhibit Akt activation in human endometrial adenocarcinoma cells. However, the detailed mechanism of action of these compounds remains to be explored.

This current study was undertaken to demonstrate and explore the ER subtype-specific action of one of the identified compounds namely, 2-[piperidinoethoxyphenyl]-3-[4-hydroxyphenyl]-2H-benzo(b) pyran (K-1) in endometrial cancer cells. Herein, we have attempted to define its anti-proliferative mechanism of action by exploring the estrogen receptor subtype-mediated action and the modulation of cell cycle regulatory proteins i.e. cyclinE1 and CDKI p21 in endometrial adenocarcinoma cells. The study shows that benzopyran compound K-1 acts as ERβ agonist in addition to the antagonistic effect on ERα..

2. Materials and methods

2.1. Compound

2-[Piperidinoethoxyphenyl]-3-[4-hydroxyphenyl]-2H-benzo(b) pyran (K-1) (Fig. 1A) were synthesized according to the methods as described earlier [19,23].

2.2. Reagents

Modified Eagle's medium (MEM)/Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma–Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Anti-ERα (HC-20), -cyclinE1, -p21, -Ki67, PCNA, cytokeratin-7 and -β-actin antibodies were purchased from Santa Cruz, CA, USA. Antibodies for β-catenin, c-myc, c-jun and c-fos were purchased from Cell Signaling Technology and antibody for ERβ (Ab-2) was purchased from Calbiochem, San Diego, CA, USA. Immuno-Blot™ PVDF membrane was purchased from Millipore, MA, USA. Reagents for western blot were obtained from GE healthcare, USA. Dual Luciferase Assay System was procured from Promega, USA.

All bacterial culture, cell culture and SDS-PAGE reagents were purchased from Sigma, USA, unless otherwise stated. 1,3,5-Tris (4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT), 17β-estradiol were purchased from Sigma, USA where as diarylpropionitrile (DPN) and ICI182,780 (Fulvestrant) (ICI) were purchased from Tocris, Ellisville, MO, USA.

2.3. Radiochemical

[2,4, 6, 7-^{3}H] Oestradiol (Specific activity-89 Ci/mmol) was procured from GE Healthcare (Amersham), UK.

2.4. Plasmids

pGL3 2× ERE pS2-luc, pSG5-mERα and pSG5-hERβ plasmids were generous gifts from Prof. M. G. Parker, Imperial Cancer Research Fund, London, UK. WWP-Luc (p21/WAF1 promoter) were obtained from Addgene, pRL-luc was purchased from Promega, USA.

2.5. Cell culture

Human endometrial cancer cell line, Ishikawa was purchased from European Collection of Cell Cultures, human endometrial cancer cells HEC1B and prostate cancer cells DU145 was purchased from American Type Culture Collection. Monkey kidney fibroblast COS-1 cell line was procured from National Center for Cell Sciences, Pune. They were maintained in MEM/DMEM supplemented with 10% fetal bovine serum (FBS). Cells were cultured at 37 °C and 5% CO_2. Prior to experiments, cells were cultured in phenol red-free MEM/DMEM supplemented with 10% charcoal stripped fetal bovine serum.

2.6. Primary cell culture of endometrial adenocarcinoma

Endometrial carcinoma samples were collected in the operating room of the Department of Obstetrics and Gynecology, Chhatrapati Shahuji Maharaj Medical University, Lucknow. A specific informed consent was obtained from each patient, and the study was approved by the local Ethics Committee. The cell isolation is based on the methods of [24]. Briefly, tissue were collected in DMEM, minced in 1-mm pieces, and incubated with 1 mg/ml collagenase and DNase (2 mg/ml) in DMEM for 2 h at 37 °C with periodic mixing. Digested tissue was mechanically dissociated through a 1-ml blue tip and resuspended in 2 ml of fresh DMEM. The cells were separated from tissue clumps and debris by filtration through an 18-mesh sterile gauze and centrifugation, washed twice with DMEM containing 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, and 2% of antibiotic–antimycotic solution (Sigma–Aldrich, USA) and then transferred into plastic culture flasks (75 cm^2, Corning, USA). Cells were incubated at 37 °C with saturating humidity and 5% CO_2. Prior to experiments, cells were cultured in phenol red-free DMEM supplemented with 10% charcoal stripped fetal bovine serum and 1% antibiotic antimycotic solution.

2.7. Competitive binding assay

Competitive binding assay was performed using charcoal adsorption method for separation of bound and free. Briefly, aliquots of human recombinant ERα or β (Panvera Corporation-Invitrogen, USA) were incubated with ^{3}H-Estradiol (2.5 nM) (Amersham Life Sciences, UK) in the presence or absence of various concentrations of test competitors for 22 h at 4 °C. Radioactivity was assessed in bound fraction in liquid scintillation counter (Beckman LS6500 model). Percent binding activity was plotted against

Please cite this article in press as: I. Fatima, et al., The anti-proliferative effect of 2-[piperidinoethoxyphenyl]-3-[4-hydroxyphenyl]-2H-benzo(b) pyran is potentiated via induction of estrogen receptor beta and p21 in human endometrial adenocarcinoma cells, J. Steroid Biochem. Mol. Biol. (2013), http://dx.doi.org/10.1016/j.jsbmb.2013.04.005