Journal of Steroid Biochemistry & Molecular Biology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

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

Estrogenic activity of isoflavonoids from the stem bark of the tropical tree *Amphimas pterocarpoides*, a source of traditional medicines

Job Tchoumtchoua[a,b,c], Maria Makropoulou[a,b], Sylvain Benjamin Ateba[c], Athina Boulaka[a], Maria Halabalaki[b], George Lambrinidis[d], Aggeliki K. Meligova[a], Jean Claude Mbanya[e], Emmanuel Mikros[d], Alexios-Leandros Skaltsounis[b], Dimitra J. Mitsiou[a], Dieudonne Njamen[c], Michael N. Alexis[a,*]

[a] *Institute of Biology, Medicinal Chemistry and Biotechnology, National Hellenic Research Foundation, Athens, Greece*
[b] *Division of Pharmacognosy and Natural Products Chemistry, Faculty of Pharmacy, University of Athens, Panepistimioupoli Zografou, 15771 Athens, Greece*
[c] *Laboratory of Animal Physiology, Department of Animal Biology and Physiology, Faculty of Science, University of Yaounde 1, P.O. Box 812, Yaounde, Cameroon*
[d] *Division of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Athens, Panepistimioupoli Zografou, 15771 Athens, Greece*
[e] *Department of Internal Medicine and Specialties, Faculty of Medicine and Biomedical Sciences, University of Yaounde 1, P.O. Box 8046, Yaounde, Cameroon*

ARTICLE INFO

Article history:
Received 31 July 2015
Received in revised form 26 November 2015
Accepted 13 December 2015
Available online xxx

Keywords:
Amphimas pterocarpoides
Isoflavonoids
Estrogenic activity
Estrogen receptor
Transactivation
HC11 cells

ABSTRACT

Various preparations of the African tree *Amphimas pterocarpoides* Harms are traditionally used to treat endocrine- related adverse health conditions. In the ovariectomized rat, the enriched in phenolics fraction of the methanol extract of stem bark of *A. pterocarpoides* acted as vaginotrophic agent of considerably weaker uterotrophic activity compared to estradiol. Evaluation of the fraction and 11 isoflavonoids isolated therefrom using Ishikawa cells and estrogen receptor (ER) isotype-specific reporter cells suggested that the estrogenic activity of the fraction could be attributed primarily to daidzein and dihydroglycitein and secondarily to glycitein. The potency-based selectivity of daidzein, dihydroglycitein and glycitein for gene expression through ERβ *versus* ERα, expressed relative to estradiol, was 37, 27 and 20, respectively. However, the rank order of relative-to-estradiol potencies of induction of alkaline phosphatase in Ishikawa cells, a reliable marker of estrogenic activity, was daidzein > dihydroglycitein >> glycitein. The considerably higher estrogenic activity of dihydroglycitein compared to glycitein could be attributed to the partial agonist/antagonist activity of dihydroglycitein through ERβ. Calculation of theoretical free energies of binding predicted the partial agonism/antagonism of dihydroglycitein through ERβ. The fraction and the isolated isoflavonoids promoted lactogenic differentiation of HC11 mammary epithelial cells at least as effectively as premenopausal levels of estradiol. This data suggests that the estrogenic activity of the fraction likely depends on the metabolism of glycitein to dihydroglycitein; that the fraction could exert vaginotrophic activity likely without challenging endocrine cancer risk more than estrogen-alone supplementation; and that the fraction's safety for the reproductive track warrants a more detailed evaluation.

1. Introduction

Postmenopausal estrogen deficiency is associated with a variety of adverse health effects, including urogenital atrophy and loss of bone mineral density, which can be partly prevented with hormone replacement therapy (HRT). HRT with estrogen and estrogen plus progestin is reportedly associated with lower and higher breast cancer risk, respectively [1] or with not higher breast cancer, irrespective of treatment [2]. Estrogens act on a variety of cells and tissues predominantly through binding to the two isotypes of ER, ERα and ERβ [3–6]. The uterus is a major target of estrogens. Administration of estrogen to ovariectomized mice

Abbreviations: AlkP, alkaline phosphatase; BW, body weight; ERα, estrogen receptor α; ERβ, estrogen receptor β; ERE, estrogen response element; E2, estradiol; EGF, epidermal growth factor; HPLC-DAD, high pressure liquide chromatography with diode-array detection; HRT, hormone replacement therapy; MTT, 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PRL, prolactin; RLU, relative light units.

* Corresponding author at: Institute of Biology, Medicinal Chemistry and Biotechnology, National Hellenic Research Foundation, 48, Vas. Constantinou Av, 11635 Athens, Greece. Fax: +30 210 7273831.
E-mail address: mnalexis@eie.gr (M.N. Alexis).

http://dx.doi.org/10.1016/j.jsbmb.2015.12.015
0960-0760/

increases uterine wet weight and proliferation of luminal epithelial cells by modulating the expression of thousands of genes [5]. These modulatory effects are predominantly mediated by an abundantly expressed ERα; ERβ, which is expressed at a considerably lower level, is known to dampen the activity of ERα [3,4,6].

Phytoestrogen-rich supplements potentially preventing or alleviating postmenopausal disorders are increasingly being consumed by menopausal women although their safety and efficacy are still a matter of debate [7–9] . Phytoestrogens are plant-derived polyphenols that in the majority of cases bind preferentially to ERβ and produce tissue-, cell- and gene promoter- specific effects partly similar to estrogen [10–13]. Recent studies have shown that ERβ-selective phytoestrogens tend to regulate apoptotic rather than proliferative genes; and that the potency and efficacy of ERβ-dependent regulation of gene expression by phytoestrogens may far exceed predictions based on ERβ-binding affinity [10,11]. Isoflavones are the most extensively studied class of phytoestrogens, genistein is an archetypal isoflavone and soy is a rich source of genistein. In the ovariectomized rat and mice models of postmenopausal bone loss, soy isoflavones were shown to display low uterotrophic and bone sparing effects [9,14]. However, clinical evidence for beneficial effects of soy remains controversial [8]. Hence, interest in screening other plants in search of effective alternatives to HRT remains high.

A. pterocarpoides Harms (Leguminoseae) is a large deciduous tree that is widespread in Central and West Africa, where it is commonly used in traditional medicine against endocrine-related disorders as well as other adverse health conditions [15]. Previous studies showed that hydro-ethanolic and aqueous extracts of the bark of *A. pterocarpoides* displayed antioxidant activity [16]. In addition we reported that in subchronic toxicity tests the methanol extract of the stem bark had no effect on rat hematological, biochemical, histological and anatomical parameters at doses lower than 300 mg/kg body weight (bw) [17]. However, hormonal effects in breast and endometrial cancer cells remained unexplored. We recently described a high resolution MS/MS approach allowing identification of isoflavonoids in a rich in phenolics fraction of the methanol extract of stem bark of *A. pterocarpoides*. As proof of the analytical concept, 11 isoflavonoids were isolated from this fraction and structurally characterized [18]. In the present study we show that in the ovariectomized rat model of estrogen deficiency the phenolic fraction acted as potent vaginotrophic agent of considerably weaker uterotrophic activity compared to estradiol; that these trophic effects could be attributed to ER agonist/antagonist activities of particular isoflavonoid constituents; that these activities could be explained by *in silico* models of binding to ERα and ERβ; and that according to specific cell-based assays the phenolic fraction as well as the isolated isoflavonoids appeared not able to challenge endocrine cancer risk more than estrogen-alone supplementation.

2. Materials and methods

2.1. Plant extract, composition of the phenolic fraction and compound isolation

Preparation of the phenolic fraction (**12**) and isolation and identification of its major constituents, namely, afrormosin (**1**), dihydroafrormosin (**2**), glycitein (**3**), dihydroglycitein (**4**), formononetin (**5**), daidzein (**6**), irisolidone (**7**), dihydroirisolidone (**8**), 7-methoxy-dihydrotectorigenin (**9**, a new isoflavonoid), afrormosin-7-*O*-β-D-glucopyranoside (**10**) and afrormosin-7-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (**11**) have been previously described [18] . LC-HRMS and HPLC-DAD methodology were used for the characterization of the phenolic fraction and the quantification of its major constituents following regression analysis. For the construction of calibration curves at least 5 different concentrations of each compound diluted in MeOH/H_2O 1:1 were prepared. Each point was at least triplicated. For the measurements, 5 mg of the phenolic fraction was diluted in MeOH/H_2O 1:1 and the injection volume was 20 μL. The isolated compounds had a purity > 95% as assessed with HPLC-DAD.

2.2. Assessment of binding affinities for ERα and ERβ

The binding affinities of **3**, **4** and **6** relative to that of estradiol (relative binding affinity, RBA) were assessed using full-length recombinant ERα and ERβ competitor assay kits (Invitrogen) and a Safire II microplate reader with fluorescence polarization detection capabilities (Tecan) as already described [19]. Briefly, we determined the concentrations of 17β-estradiol, **3**, **4** and **6** that inhibited the binding of the fluorescent estrogen ES2 (Invitrogen) to ERα and ERβ by 50% (IC_{50}) and used them to calculate the RBAα and RBAβ, respectively, as previously described [19].

2.3. Cell lines, tissue culture media and chemicals

MCF-7 and MDA-MB-231 human breast adenocarcinoma cells were purchased from ATCC. Ishikawa human endometrial adenocarcinoma cells were from ECACC. The cells were cultured as recommended by the suppliers. HC11 mouse mammary epithelial cells were kindly provided by Professor Dr Bernd Groner. The cells are known to model mammary cell differentiation upon exposure to lactogenic hormones [20] . HC11 cells were cultured in RPMI 1640 medium (GlutaMAX™, Gibco) supplemented with 10% FBS (Biosera), 5 μg/mL insulin and 10 ng/mL EGF (Peprotech) and were subcultured before reaching confluence. MCF-7:D5L cells and HEK:ERβ cells were generated and cultured as previously described [21] . MCF-7:D5L cells, a clone of MCF-7 cells, are stably transfected with the estrogen response element (ERE)-endowed reporter plasmid pERE-Gl-Luciferase. HEK:ERβ cells, a clone of HEK-293 human embryonic kidney cells, are stably transfected with an expression plasmid coding for the full-length human ERβ as well as the ERE-endowed reporter plasmid pERE-tk-Luciferase. Unless specified otherwise, cell culture media, fine chemicals and hormones, including daidzein (D7802, ≥98%), were from Sigma-Aldrich and FBS from Invitrogen. Glycitein (≥95%, HPLC) was from Extrasynthese S.A. and the estrogen receptor (ER) degrader ICI182,780 (Fulvestrant) was from Tocris Bioscience.

2.4. Assessment of ER-regulated luciferase expression

Regulation of ERE-dependent luciferase gene expression in MCF-7:D5L and HEK:ERβ cells was assessed as already described [22]. Briefly, the cells were plated in 96-well plates at a density of 12,000 cells per well in MEM (MCF-7:D5L cells) or DMEM (HEK:ERβ cells) devoid of phenol-red and supplemented with 1 μg/mL insulin and 5% DCC-FBS *i.e.* FBS treated with 10% dextran-coated charcoal to remove endogenous steroids. Three days after plating the cells were exposed for 18 h to test compounds in the absence or presence of 1 nM estradiol and/or 1 μM ICI182,780., The compound diluent (DMSO, vehicle) was kept to a final concentration ≤0.2%. Luciferase expression was assessed using the Steady-Glo Luciferase Assay System (Promega) and a Safire II microplate reader (Tecan).

2.5. Assessment of alkaline phosphatase expression in Ishikawa cells

Regulation of alkaline phosphatase (AlkP) expression of Ishikawa cells was assessed using 96-well plates and 12,000 cells per well in phenol-red-free MEM supplemented with 1 μg/mL insulin and 5% DCC-FBS. 24 h after plating, the cells were exposed to test compounds in the absence or presence of 0.1 or 1 nM

Please cite this article in press as: J. Tchoumtchoua, et al., Estrogenic activity of isoflavonoids from the stem bark of the tropical tree *Amphimas pterocarpoides*, a source of traditional medicines, J. Steroid Biochem. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jsbmb.2015.12.015