Steroids 105 (2016) 42–49

Contents lists available at ScienceDirect

Steroids

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

Licorice root components in dietary supplements are selective estrogen receptor modulators with a spectrum of estrogenic and anti-estrogenic activities

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ARTICLE INFO

Article history:
Received 17 August 2015
Received in revised form 9 November 2015
Accepted 18 November 2015
Available online 26 November 2015

Keywords:
Estrogen receptor
Selective estrogen receptor modulator
Botanical estrogens
Licorice root estrogens

ABSTRACT

Licorice root extracts are often consumed as botanical dietary supplements by menopausal women as a natural alternative to pharmaceutical hormone replacement therapy. In addition to their components liquiritigenin (Liq) and isoliquiritigenin (Iso-Liq), known to have estrogenic activity, licorice root extracts also contain a number of other flavonoids, isoflavonoids, and chalcones. We have investigated the estrogenic activity of 7 of these components, obtained from an extract of *Glycyrrhiza glabra* powder, namely Glabridin (L1), Calycosin (L2), Methoxychalcone (L3), Vestitol (L4), Glyasperin C (L5), Glycycoumarin (L6), and Glicoricone (L7), and compared them with Liq, Iso-Liq, and estradiol (E2). All components, including Liq and Iso-Liq, have low binding affinity for estrogen receptors (ERs). Their potency and efficacy in stimulating the expression of estrogen-regulated genes reveal that Liq and Iso-Liq and L2, L3, L4, and L6 are estrogen agonists. Interestingly, L3 and L4 have an efficacy nearly equivalent to E2 but with a potency ca. 10,000-fold less. The other components, L1, L5 and L7, acted as partial estrogen antagonists. All agonist activities were reversed by the antiestrogen, ICI 182,780, or by knockdown of ERα with siRNA, indicating that they are ER dependent. In HepG2 hepatoma cells stably expressing ERα, only Liq, Iso-Liq, and L3 stimulated estrogen-regulated gene expression, and in all cases gene stimulation did not occur in HepG2 cells lacking ERα. Collectively, these findings classify the components of licorice root extracts as low potency, mixed ER agonists and antagonists, having a character akin to that of selective estrogen receptor modulators or SERMs.

1. Introduction

Estrogens working through estrogen receptors (ERs) exert effects on diverse target cells and tissues, so that the sharp decline in ovarian estrogen production at menopause broadly impacts the health of women [1–3]. Many menopausal women take botanical dietary supplements as an alternative to pharmaceutical hormone replacement therapy (HRT), to achieve relief from menopausal symptoms that include hot flushes, vaginal atrophy, bone loss, and changes in cardiovascular and metabolic function. While pharmaceutical estrogens used in HRT are effective in alleviating vasomotor symptoms and have beneficial effects on bone, cardiovascular and metabolic health, they may pose risks by exacerbating breast and uterine cancer development and/or progression.

Abbreviations: E2, estradiol; Iso-Liq, isoliquiritigenin; Liq, liquiritigenin; RBA, relative binding affinity; SERM, selective estrogen receptor modulator.

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http://dx.doi.org/10.1016/j.steroids.2015.11.006
0039-128X/

Licorice root extracts are frequently used in dietary supplements and are believed to contain estrogenic components that might, in principle, provide a spectrum of beneficial effects with reduced stimulation of the breast and uterus compared with that of the endogenous hormone estradiol (E2) or the pharmaceutical estrogens used in HRT [4,5]. However, much is still not known about the mechanisms by which licorice root components act and whether they have the same or a different spectrum of activity from that of ovarian or pharmaceutical estrogens. Also of importance is whether any of the estrogenic components in licorice extracts are of sufficient potency that they would be efficacious at the doses typically provided by licorice botanical supplements consumed by women.

Recent studies by us and others have characterized the estrogenic activity of a major licorice root component, liquiritigenin (Liq) [4,6,7]. Our whole genome RNA-seq gene expression and cell proliferation studies comparing E2 and Liq revealed that the estrogenic activity of Liq was weaker than that of E2 and that some genes showed distinctly different regulation of expression (stimulation and/or suppression) by Liq versus E2 [6,7]. Licorice root extracts, as used in dietary supplements, contain variable amounts of a number of compounds in addition to Liq that could have estrogenic and/or anti-estrogenic activities, and might contribute in a differential manner to the overall potency and activity of these dietary supplements. Therefore, we have characterized chemically and biologically the activity of these components in ER-containing breast cancer cells (MCF7) and liver hepatoma (HepG2-ER) cells, these being representative of two target tissues in which estrogens are known to elicit important physiological effects, including enhanced breast cancer cell proliferation (MCF7) and beneficial changes in cell metabolism (liver cells).

In this study, we report on the estrogenic and anti-estrogenic activities of seven components of licorice root, and we compare these with each other and with E2, as well as with Liq and Iso-Liq, the two most widely studied components in licorice root extracts. Our findings reveal that four of the seven components have significant estrogen agonist activity that can contribute, along with Liq and Iso-Liq, to the overall composite estrogen activity of licorice root extracts. Of interest, three of the components are largely ER antagonists. Our observations reveal the biological activities present in multi-component dietary supplements such as licorice root extracts. Furthermore, they highlight the need to standardize the preparation of licorice root extracts in dietary supplements, because the manner in which supplements are prepared could greatly impact the relative proportions of the different components and hence the spectrum of biological activities in different licorice root dietary supplement preparations.

2. Experimental

2.1. Ligands and cell culture

Estradiol was from Sigma (St Louis, MO). Liquiritigenin and isoliquiritigenin, and the antiestrogen ICI 182,780 were from Tocris Bioscience (Bristol, UK). All compounds were checked for identity and purity by mass spectrometry and NMR. MCF-7 cells (ATCC) were cultured in DMEM, Dulbecco's Minimal Essential Medium (Gibco/Life Technologies, Grand Island, NY), supplemented with 5% calf serum (HyClone, Logan, UT) and 100 μg/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA), as previously described [7,8]. For estrogen-free experiments, the cells were seeded in phenol red-free DMEM (Gibco/Life Technologies) plus 5% charcoal–dextran-treated calf serum for 3 days before treatments with compounds were initiated. HepG2 cells (ATCC) and HepG2 cells stably expressing ERα (HepG2 + ERα cells, kindly provided by David J. Shapiro, University of Illinois at Urbana-Champaign) were grown as described [9,10]. The siRNA experiments for knockdown of the endogenous ERα in MCF-7 cells were performed as previously described and resulted in knockdown of ERα mRNA and protein by greater than 95% [11]. siERα sequences (Dharmacon, Lafayette, CO) were forward, 5′-UCAUCGCAUUCCUUGCAAAdTdT-3′, and reverse, 5′-UUUGCAAGGAAUGCGAUGAdTdT-3′ [11].

2.2. Preparation of licorice root extract and isolation and purification of licorice root components

Licorice root (*Glycyrrhiza glabra*) powder (750 g) was extracted with methanol (3 × 3 L for 20 h) using a percolator. The solvent was removed under reduced pressure at 48 °C to yield the dried extract (107 g). The pure compounds, Liq (0.378% yield), Isoliq (0.23%), L1 (0.514%), L2 (0.006%), L3 (0.027%), L4 (0.027%), L5 (0.021%), L6 (0.014%), and L7 (0.005%) were obtained from the methanolic extract of licorice by column chromatography over normal silica gel, reversed phase silica (RP-18), and Sephadex LH-20, as well as preparative thin layer chromatography (PTLC). Product integrity and purity of all 9 flavonoids were characterized using various analytical techniques such as NMR and mass spectrometry.

2.3. Relative binding affinity assays

Relative binding affinities were determined by a competitive radiometric binding assay as previously described [12,13], using 2 nM [^{3}H]-estradiol as tracer ([2,4,6,7-^{3}H]-estra-1,3,5(10)-triene-3,17β-diol, 70–115 Ci/mmol, Perkin Elmer, Waltham, MA), and purified, full-length, human ERα and ERβ purchased from PanVera/Invitrogen (Carlsbad, CA). Incubations were for 18–24 h at 0 °C. Hydroxyapatite (BioRad, Hercules, CA) was used to absorb the receptor–ligand complexes, and free ligand was washed away. The binding affinities are expressed as relative binding affinity (RBA) values with the RBA of estradiol set to 100%. The values given are the average ± range or SD of two to three independent determinations. Estradiol binds to ERα with a K_d of 0.2 nM and to ERβ with a K_d of 0.5 nM, as previously determined [12].

2.4. RNA isolation and real-time PCR

Total RNA was isolated from cells using TRIzol (Invitrogen), RNA samples were reverse transcribed by MMTV reverse transcriptase (New England Biolabs, Ipswitch, MA), and real-time PCR was carried out on the ABI Prism 7900HT instrument using SYBR Green PCR Master Mix (Roche), as described previously [14].

2.5. Statistical analysis

Statistical analyses used one-way ANOVA with Bonferroni's Multiple Comparison Test or two-way ANOVA with Bonferroni posttest and used GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA). Data are expressed as mean ± SD and $P < 0.05$ was assigned as statistically significant.

3. Results

3.1. Preparation of licorice root extracts and characterization of chemical components and their binding affinities for estrogen receptors

An overview of the extraction and purification protocol is presented in Fig. 1 and described in the Methods section. All compounds were checked for purity and identity by mass