INTRODUCTION

Estrogen receptors (ERs) are ligand-dependent transcription factors that facilitate the regulation of numerous physiological programs such as development, metabolism and homeostasis. The predominant endogenous ligand for ERs is estrogenic compound E2. It is synthesized and secreted mainly by the ovaries in females, and testes and adrenal cortex in males. Nevertheless, E2 is produced in a number of other tissues including adipose tissue, liver, skin, pancreas and brain [1,2,3]. Since ERs are expressed in numerous tissues, estrogens exhibit complex multisystemic effects, and are, consequently, implicated in the development and progression of various diseases.

While E2 is the natural ligand for ERs, these receptors can also be activated by various endogenous and exogenous ligands capable of binding ERs and inducing diverse receptor conformations that affect estrogen-dependent gene regulation. These ER ligands are acting by promoting (i.e., agonists) or blocking estrogenic activity (i.e., antagonists), modifying a variety of biological effects. Understanding agonistic/antagonistic features of a ligand demands the recognition of ligands interference with receptor activity at various levels: from the stability of ER-transcriptional complex, through ER's homo- and heterodimerization ability to its interaction with DNA response elements. Since ligand characteristics are highly diverse, a small difference in a structure can cause critical difference in outcome and add intricacy to overall ER-mediated effects. The most famous examples are selective estrogen receptor modulators (SERMs) that are targeting the ER for the treatment of endocrine disorders [4,5]. Although SERMs bind the same site on ER as E2 they exert selective agonist or antagonist activity in different target tissues as a consequence of unique SERM-receptor conformations.

A distinctive group of ER ligands are phytoestrogens, a form of dietary plant-derived estrogen-mimicking compounds. Phytoestrogens could affect the function of estrogens in different ways. They can modulate estrogen synthesis and metabolism or transport of the estrogens through the body [6,7]. But the best examined phytoestrogens are those acting as ER ligands i.e., binding directly to ERs and competing with endogenous estrogens [8]. The overall effect of phytoestrogens will depend upon sex, age and health status of the consumer, as well as dose, type and synergistic interactions among consumed compounds, the level of endogenous estrogens and ER isoforms presence [9,10,11].
ESTROGEN RECEPTORS: STRUCTURE AND MECHANISMS OF ACTION

Most estrogen hormone effects are mediated by two different ERs, ERα and ERβ [12,13]. ERα and ERβ are encoded by different genes; the human ERα gene is located on chromosome 6q25.1 [14], whereas the human ERβ gene is located on chromosome 14q23.2 [15]. ERα and ERβ exert disparate biological roles, as evidenced by their distinct intracellular and tissue distribution patterns [16,17], different gene regulation patterns [18] and different phenotypes detected in ERα and ERβ knockout mice [19].

As members of the nuclear receptor superfamily [20], ERα and ERβ have a general multidomain structure. The ERs consist of six functional domains: a variable, N-terminal A/B domain which contains activation function-1 (AF-1) that can activate gene transcription in a constitutive manner; a central C domain known as the DNA-binding domain (DBD) responsible for binding to specific DNA sequences known as estrogen response elements (ERE); linker or hinge region between the LBD and DBD known as D domain; and a C-terminal E/F domain, which contains the ligand-binding domain (LBD), dimerization interfaces and activation function-2 (AF-2), that serves as a binding site for coregulatory proteins [21]. While DBD in ERα and ERβ is highly conserved exhibiting 95% homology, the LBD is flexible, with only partial homology (55%).

ERs regulate physiological processes through two major mechanisms. In the conventional genomic pathway of estrogen action, multistep process responsible for ER-mediated gene regulation takes place [22,23]. This mode of action begins by binding of the ligand to the ER LBD. This event produces a conformational change in ER that depends strongly on the nature of the ligand and leads to the receptor dimerization, and binding to the ERs [24]. The ligand binding also induces a conformational change within the ER LBD allowing coregulatory proteins to be recruited [25,26] which culminates in the modulation of target gene expression in response to estrogens. Nevertheless, ERs can regulate transcription without binding directly to DNA. ERs in such cases are acting through protein-protein interactions with a transcription factor complex that contacts the DNA [27].

The second pathway of ER action is the non-genomic mechanism which mediates rapid signaling events [28,29]. In this pathway, initiated at the cell membrane, estrogens bind to membrane-associated ERs - ERα, ERβ or G protein-coupled estrogen receptor (GPER1), and cause non-genomic effects. These actions include the mobilization of intracellular calcium [30], the stimulation of adenylate cyclase activity and cAMP production [31,32] and the activation of various protein-kinase cascades [33].

It is important to recognize that different mechanisms of ER actions are co-dependent, overlapping and even converging at same target genes. Namely, non-genomic pathway of ER action may indirectly regulate gene expression, through activation of signaling pathways that act on target transcription factors.

INTERACTION OF PHYTOESTROGENS WITH ERs

Phytoestrogens are plant-derived substances with estrogenic properties. Although they have been used in traditional medicine for a long time, the interest in phytoestrogens is intensified due to the increased amount of information on their effects on human health, as well as better understanding of estrogen action in general. As a consequence of their estrogenic activity, phytoestrogens have been ascribed beneficial health effects such as the alleviation of menopausal symptoms, prevention of osteoporosis, reduced risks of cardiovascular disease, obesity, and even breast, endometrial and prostate cancer. However, adverse effects of phytoestrogens have also been reported [34]. As already mentioned, majority of research in phytoestrogen signaling has focused on their role as ER ligands, i.e. their ability and the ability of their metabolites to interact and activate ERs.

Phytoestrogens are highly diverse group found in more than 300 plant species [35] including a wide variety of human foods such as soy products, legume seeds and dairy products [36,37]. According to their chemical structure phytoestrogens are nonsteroidal polyphenols. Their structural similarity with estrogens (e.g., the presence of phenolic rings) is a requirement for phytoestrogen to bind ER. Essentially, they are classified in two groups: flavonoids and non-flavonoids [38]. Flavonoids have the fifteen-carbon skeleton (C6–C3–C6) which consists of two benzene rings (A and B) and a heterocyclic pyran structure (C). Best characterized groups of flavonoids are isoflavones, coumestans and prenylflavonoids (Figure 1). This classification is based on structural differences [39] and modifications in the skeleton and/or the chemical groups of flavonoids [Reviewed in detail in 40]. Non-flavonoids consist of naturally occurring phenolic acids, either hydroxybenzoic acid (C6-C1 structure) or hydroxycinnamic acid (C6-C3 structure) [39]. Main representatives of non-flavonoid phytoestrogens are lignans and stilbenes (Figure 2).

In plants, the phytoestrogens perform mainly a protective and defense functions exerting anti-parasitic, antibacterial, antiviral and fungistatic effects. They also act as antioxidants, protecting the plant from harmful effects of sunlight [41].

In the human body, the phytoestrogens are rarely active as free molecules. They are metabolized both by tissue enzymes and gastrointestinal microbiota, either
prior to absorption or during enterohepatic circulation. Notably, individual phytoestrogen metabolites have often higher ER binding affinity than phytoestrogen itself. Most of ingested phytoestrogens are unabsorbed in the proximal intestine \[42,43\] but reach the colon where they are exposed to microbiota and undergo metabolism \[44,45\]. Gut microbiota is assumed to play an essential role in the metabolism of phytoestrogens in humans by impacting the degree to which they are absorbed, metabolized and degraded.
Thus, the differences in an individual’s distinctive gut microbiota, which is determined by many factors including diet, bowel disease, stress, gender, age and genetics, may play an important role in the bioavailability of phytoestrogens and their metabolites, and the outcomes of any subsequent biological response.

**MOLECULAR MECHANISMS OF PHYTOESTROGEN ACTION**

Since the majority of phytoestrogen effects are mediated through ERs, it is evident that small differences in phytoestrogen/ER structural conformation will cause significant differences in phytoestrogen biological activity. Thus, the difference between the actions of particular phytoestrogens can be explained in large part by differences in their ability to influence ER transcriptional functions.

The transcriptional, and consequently physiological response to a phytoestrogen depends on a number of factors, including: 1) differences in ER subtype affinity and selectivity, 2) differences in recruitment of coregulatory proteins and/or 3) differences in promoter context, i.e., altered specificity and affinity of the DNA response elements through which the ER is acting. As a result, phytoestrogens have a high potential for modifying all estrogen-dependent biological activities and cell signaling pathways in all imaginable ways [Reviewed in detail in 40].

One of the most extensively studied phytoestrogens is the isoflavone genistein that corresponds to the chemical name ‘4’,5,7-Trihydroxyisoflavone’. It is typically found in legumes, such as soybeans and fava beans, and is being metabolized by endogenous phase I and phase II enzymes as well as by the gut bacteria leading to production of metabolites with altered bioactivity [46,47]. Majority of its effects at nutritionally relevant concentrations are ER-mediated [48]. Furthermore, genistein predominantly employs ER classical genomic mechanism [49].

Insight into the genistein mechanism of action is connected with understanding the differences in ERα vs ERβ action (Table 1). Although ERα and ERβ are mediating multiple biological effects in a similar manner, their actions are sometimes resulting in entirely opposing effects. Namely, ERs are modulators of cell growth and differentiation but ERα stimulates cell growth and proliferation, while ERβ is associated with cell differentiation and tumor suppression [59]. On the other hand, both ERα and ERβ are equally effective in transcriptional repression of inflammatory genes responsible for osteoporosis [60] and other beneficial ER effects. Consequently, much effort has been applied in developing and/or discovering ERα and ERβ selective ligands that will have selective clinical effects and uncouple beneficial actions of ERs from deleterious effects.

### Table 1. ER subtype specificity of representative phytoestrogens.

<table>
<thead>
<tr>
<th>Name</th>
<th>Class</th>
<th>ER specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FLAVONOIDS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>genistein</td>
<td>isoflavones</td>
<td>ERβ &gt; ERα</td>
<td>[50]</td>
</tr>
<tr>
<td>daidzein</td>
<td>isoflavones</td>
<td>ERβ &gt; ERα</td>
<td>[49,51]</td>
</tr>
<tr>
<td>biochanin A</td>
<td>isoflavones</td>
<td>ERβ &gt; ERα</td>
<td>[52]</td>
</tr>
<tr>
<td>quercetin</td>
<td>flavonol</td>
<td>ERβ &gt; ERα</td>
<td>[53-55]</td>
</tr>
<tr>
<td>coumestrol</td>
<td>coumestans</td>
<td>ERβ &gt; ERα</td>
<td>[50]</td>
</tr>
<tr>
<td>8-prenylnaringenin</td>
<td>prenylflavonoids</td>
<td>ERα ≥ ERβ</td>
<td>[56,57]</td>
</tr>
<tr>
<td><strong>NON-FLAVONOIDS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>enterolactone</td>
<td>lignans</td>
<td>ERα &gt; ERβ</td>
<td>[50,53,58]</td>
</tr>
<tr>
<td>resveratrol</td>
<td>stilbenes</td>
<td>ERα &gt; ERβ</td>
<td>[50,53]</td>
</tr>
</tbody>
</table>

Genistein, like most of the phytoestrogens identified so far, is ERβ-selective, and as such may act as natural SERM [61]. The genistein selectiveness is direct consequence of difference in genistein relative binding affinity given that genistein binds with a 20-fold greater affinity for ERβ than ERα [49]. Thus, genistein offers a great potential as ERβ selective ligand, which is avoiding cancer-promoting effects of ERα, but keeping beneficial ERβ-mediated effects.

On the molecular level, genistein modulates various steps of cell cycle through ER, in order to induce the desired antiproliferative effect. The regulation of cell proliferation by genistein implores multiple mechanisms leading to G2/M cell cycle arrest. These mechanisms are primarily driven by the regulation of crucial cell cycle regulatory genes including several cyclins, among them cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs). Another genistein-mediated mechanism engaged in G2/M cell cycle arrest involves the Ras/MAPK/activator protein-1 signaling pathway and results in the suppression of the transcription factors Cdk1, cyclin B1, and Cdc25C [62].

In other context and as a consequence of multiple levels of action, genistein has antioxidant effect. There is direct antioxidant effect of genistein that derives from its capacity to act as a ROS scavenger [63,64]. Furthermore, genistein through ER stimulates the expression of the antioxidant enzymes manganese superoxide dismutase, glutathione peroxidase and catalase [65,66].

However, it will be way too simple to consider the effects of genistein (or any other phytoestrogen or ER ligand in general) exclusively in the light of cell cycle regulation and/or antioxidant effects. Multiple mechanisms producing antiangiogenic, antimetastatic, anti-inflammatory and anti-amyloid-β activities are not
going to be considered here [67,68]. Besides, most of our knowledge about phytoestrogens is the result of the studies concentrated on one compound at a time, extremely unlikely context to be found in reality.

In fact, we are constantly exposed to a myriad of compounds that are competing for ERs and producing wide range of effects depending on the fine balance between presence of ERs in tissues, concentrations of endogenous estrogens and the content and dosages of consumed phytoestrogens. Above all, it is necessary to acknowledge synergistic interactions among estrogenic compounds that are as significant as the effects of each of them separately. Brilliant example is MF101 (Menerba) - the herbal extract from number of plants, with distinctive estrogenic activity. MF101 was studied as a potential treatment for hot flashes in postmenopausal women. MF101 formula was based on the use of botanical supplements exploited in Traditional Chinese Medicine for the treatment of menopausal symptoms [69].

What makes it interesting, MF101 is crude herbal extract containing 22 individual plants and multitude of the compounds that synergistically act in order to produce unique effect [70,71]. Despite such a complexity MF101 is ERβ selective. Unlike genistein and most of the phytoestrogens whose receptor subtype selectivity is a consequence of difference in binding affinity, MF101 binds to both ERα and ERβ with equal affinity [70]. In this case, ERβ selectivity of MF101 is due to the selective recruitment of cofactors in respect to difference in conformational change produced by MF101 in ERα vs ERβ [70].

Thus, MF101-mediated physiological effects such as alleviation of menopausal symptoms like hot flushes [72] with no induction of proliferative events are likely the consequence of synergistical action of multiple compounds. We also shouldn’t forget that prescription drugs are often used alongside either phytoestrogen-containing food or herbal supplements with a hope of synergistic beneficial effects but with a chance of unwanted competitive interactions.

CONCLUSIONS

Recent advances in our understanding of the phytoestrogens as effective ER modulators have spurred substantial interest in their potential to serve as therapeutic agents. Initial studies focused on the conformational binding effects of various ligands that would be beneficial for targeting ERs. However, the standard picture of the particular compound that has beneficial effect on particular condition turned out to be oversimplified. Multiple factors converge on the ER transcriptional regulation to determine the final response. The obvious determinants are variability in dietary phytoestrogen intake: frequency, timing and composition of consumed phytoestrogens, as well as substantial inter-individual differences in the metabolic and pharmacokinetic processing of ingested phytoestrogens. As already underlined, the presence and distribution of ER subtypes and transcriptional cofactors in different tissues and cell types are adding to complexity of the outcome. Finally, the amount and type of endogenous hormone which will compete for the binding of the ERs with exogenous ligands will determine the overall effect. Nevertheless, such interplay allows ERs to respond dynamically to any ligand.

So, in spite of the extraordinary expansion of the phytoestrogen studies in the last decades, the level of complexity and context-dependent nature of its actions are a real challenge for the future.

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Fitoestrogeni kao ligandi estrogenskog receptora

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Kratak sadržaj
Estrogenski receptori (ER) su ligandom regulisani transkripcijski faktori koji modulisu esencijalne programe transkripcije aktiviranjem ili reprimiranjem ciljane ekspresije gena. S obzirom na uticaj ER signalizacije na razvoj, metabolizam i fiziologiju, izmenjeno funkcionisanje ER leži u osnovi mnogih poremećaja, zbog čega su ER odavno prepoznati kao biološke i farmaceutske mete od izuzetne važnosti. Iako je 17b-estradiol (E2) glavni endogeni ER ligand, postoji mnoštvo jedinjenja, uključujući fitoestrogene, koja oponašaju estrogene i takođe aktiviraju ER. Fitoestrogeni su sposobni da se vežu za ER i tako utiču na signalne puteve regulisane estrogenom, izazivajući koliko korisne toliko i štetne posledice. U ovom radu dat je sažet pregled dosadašnjih saznanja iz oblasti fitoestrogena u funkciji alternativnih ER liganada.

Ključne reči: Estrogenski receptor; fitoestrogeni; MF101; genistein.