

Glyceollins, a Novel Class of Antiestrogenic Phytoalexins

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Abstract

Glyceollins, a group of novel phytoalexins isolated from activated soy, have demonstrated unique *in vitro* and *in vivo* antiestrogenic activity. The soybean plant under stress produces a mixture of glyceollins I, II, and III that bind to the estrogen receptor (ER) and inhibit estrogen-induced tumor progression. In further *in vitro* studies, the glyceollin mixture exhibits potential antiestrogenic, therapeutic activity preventing estrogen-stimulated tumorigenesis and displaying a differential pattern of gene expression from tamoxifen. By isolating the individual glyceollin isomers, we identified the active antiestrogenic component using competition binding assays with human ER α and estrogen-responsive element-based luciferase reporter assays. We identified glyceollin I as the active antiestrogenic component of the mixture. Ligand-receptor modeling (docking) of the isomers within the ER α ligand binding cavity demonstrated a unique type II antiestrogenic confirmation adopted by glyceollin I, but not isomers II and III. Glyceollin I treatment in 17 β -estradiol-stimulated MCF-7 breast cancer cells and BG-1 ovarian cancer cells resulted in a novel inhibition of ER-mediated gene expression and cell proliferation/survival. Glyceollin I may represent an important component of a phytoalexin-enriched food (activated) diet in terms of chemoprevention as well as a novel therapeutic agent for hormone dependent tumors.

Keywords: Estrogen receptor; Antiestrogen; Selective estrogen receptor modulator (SERM); Phytoestrogen; Phyto-SERM; Phytoalexin; Glyceollins; Glycinol; Pterocarpin; Activated soy; Breast cancer

Introduction

Antiestrogen therapy such as tamoxifen has been the standard treatment of choice for pre-menopausal women with estrogen receptor (ER) positive breast cancer. Tamoxifen is a selective estrogen receptor modulator (SERM) which competes with estrogen for binding to the estrogen receptor. Although it acts as an antagonist in the breast, it displays selective agonist activity in other tissues such as uterus and bone. This activity in bone of post-menopausal women has been associated with a decreased risk of osteoporosis. Unfortunately agonistic activity in the uterus leads to an increased risk of endometrial cancer. Additionally the progression or development of tamoxifen resistance in breast cancer remains a problem in treatment of ER positive disease. As such, additional antiestrogens have been employed in the treatment of breast cancers. The pure antiestrogen, fulvestrant, is indicated for the treatment of hormone receptor-positive metastatic breast cancer in postmenopausal women with disease progression following antiestrogen therapy. In contrast to tamoxifen, there is no agonist activity associated with its use. It competes with estrogen for binding to the ER and causes ER degradation. Aromatase inhibitors (AI) on the other hand, target ER function indirectly by blocking the conversion of adrenal androgens to estrogen in the peripheral tissues of postmenopausal women. However, due to limitations associated with endocrine therapy, such as acquired resistance, there is a need for the development of new ER targeting therapeutic agents.

The discovery that plants produced hormonally active phytochemicals has changed our understanding of the relationship between diet and human health. Over 50 years ago it was first demonstrated that some plants contain distinct families of small molecule phytochemicals, which possess weak estrogenic and/or antiestrogenic

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activity (1). Also known as phytoestrogens, these compounds exhibit weak estrogenic activity on the order of 10^{-2} – 10^{-3} that of 17β -estradiol, but may be present in the body in concentrations 100-fold higher than endogenous estrogens (2). Although the function of phytoestrogens are not completely understood, observations of the estrogenic activity associated with phytoestrogens dates back to the 1950s when it was discovered that equol, the metabolite of daidzenin, was responsible for the reduced reproductive capacity of sheep grazing on clover. These natural products are plant chemicals that function as fungicides, herbivore deterrents, plant hormone regulators and ultraviolet radiation plant protectants. Phytoestrogens can be divided into three main classes: isoflavones (genistein and daidzein), coumestans (coumestrol) and lignans (enterodiol and enterolactone). They are polyphenols structurally similar to the steroid hormone 17β -estradiol (E_2) and compete with the endogenous hormone for binding to the estrogen receptor (ER) thus reducing the hormonal effect of endogenous estrogens that are much more potent than phytoestrogens (3,4).

It has been shown that the role of phytoestrogens and consumption of phytoestrogen-rich foods such as soy containing isoflavones has a protective role in the prevention of breast cancer. Asian women who consume a traditional low-fat soy diet have a four- to six-fold lower risk of developing breast cancer when compared to women living in the industrialized Western world (5-9, 2). Many of the health benefits of soybean have been attributed, in part, to the presence of isoflavones (7, 2, 10, 11-13). Isoflavones are considered phytoalexins, which are low molecular weight antimicrobial compounds. They are synthesized *de novo* and accumulate in different plant tissues in response to stress, physical stimuli, or infectious agents (14-19). In soybean, several significant changes in isoflavone concentration occur in response to stress or elicitor treatment. The predominant phytoalexins produced in soybeans are glyceollins I, II and III, and these compounds demonstrate antifungal activity against several plant pathogens. Although a recent publication has demonstrated that glyceollins possess estrogenic activity, (20) research in our laboratory has predominately focused on the antiestrogenic activity of the glyceollins *in vitro* (21) and *in vivo* (22, 23). Taken together, our studies and others have provided a rationale for the investigation of various nutritional strategies designed to target breast cancer treatment and prevention.

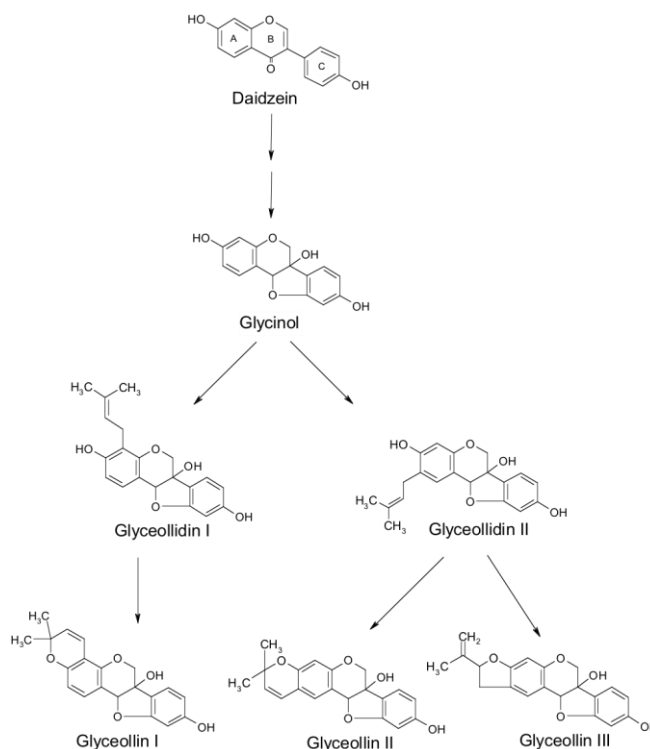


Figure 1. Flavonoid pathway showing the biosynthetic route from daidzein to glyceollins I–III.

Glyceollin Biosynthesis in Soy

The glyceollins are pterocarpan phytoalexins that can be induced at high concentrations as a mixture consisting of three isomers, Glyceollins I, II and III, when soybean cotyledons are stressed by infection with *Aspergillus* (24). Numerous other stress factors and physical stimuli induce phytoalexin accumulation, including wounding, freezing and UV light exposure (15). In addition, compounds known as elicitors, either biotic or abiotic, can stimulate the biosynthesis of phytoalexins. The activation of soy yields an increase in the production of glyceollins I, II and III. The structures and biosynthetic pathway of glyceollins I, II and III are shown in Figure 1. Glyceollins are derived from the precursor molecule, daidzein through several intermediate structures including glycinol. Glycinol, which we have previously shown to be estrogenic, is derived from daidzein via a pterocarpan by cyclization and 6 α -hydroxylation (25). For the biosynthesis of the glyceollins, glycinol is prenylated to produce glyceollidin I and II, followed by cyclization into the corresponding glyceollins (26). Given that specific features of glyceollins remained unclear, we were initially interested in determining the exact nature of its properties as they could represent an

important component of both the beneficial and/or detrimental effects on human health.

Glyceollins Exhibit Antiestrogenic Activity

Our initial studies published nearly a decade ago identified glyceollins as unique soy phytochemicals that had not previously been assessed for antiestrogenic or estrogenic activity. Glyceollins, unlike the observed estrogenic effects of coumestrol, daidzein, and genistein were demonstrated to mediate their antiestrogenic effects in breast cancer cells through both ER α and ER β , however competition binding assays revealed a greater affinity for ER α (21). Additional studies with MCF-7 cells revealed that glyceollins suppressed both E₂-mediated proliferation (21). This suggested glyceollins as well as other phytoalexins may represent an important component of the health effects of soy-based foods. Not only were the antiestrogenic effect of glyceollin observed in vitro, but in vivo studies demonstrated the potential of glyceollins to suppress human breast and ovarian carcinoma tumorigenesis by 53.4% and 74.1%, respectively (22). The antitumorigenic effects also correlated with a reduction of the intratumoral E₂-induced progesterone receptor expression (PgR). Unlike tamoxifen, glyceollin did not exhibit estrogenic effects on uterine morphology. To further investigate the idea that dietary soy enriched in glyceollins may have distinct estrogen modulating properties compared with standard soy, a postmenopausal primate model was designed to evaluate the short-term effects of glyceollin-enriched soy protein and standard soy enriched protein isolate (SPI) in combination with estrogen (23). Results revealed mean serum glyceollin concentrations at 4 h postfeeding of 134.2 ± 34.6 nmol/L in the glyceollin group and negligible in the SPI control group ($P = 0.0007$) (23). Breast proliferation was significantly increased in the control group (+237%, $P = 0.01$) but not in the SPI group (+198%, $P = 0.08$) or glyceollin group (+36%, $P = 0.18$). Intramammary gene expression of trefoil factor 1 (pS2) and PgR, two markers of estrogen receptor activity in breast epithelium, were also significantly higher in the control ($P < 0.05$ for both) but not in the glyceollin group. Taken together, these findings suggested soybean glyceollins are natural compounds with potential estrogen-modulating properties in the breast. In addition to the demonstration that glyceollins are novel antiestrogenic therapeutic agents that may be useful in the prevention and/or treatment of breast and

ovarian carcinoma, glyceollins like genistein inhibit LNCaP prostate cancer cell growth (27). The mechanism of inhibition was demonstrated to be through inhibition of G1/S progression which correlated with an up-regulation of cyclin-dependent kinase inhibitor 1 A and B mRNA and protein levels. Interestingly, these findings appeared to be due, in part, to the effects of the glyceollins on an ER-mediated event and not an androgen-receptor mediated event. Considering our in vitro and in vivo evidence, the glyceollins inhibitory effects on breast, ovarian and prostate cancer cells warrant further consideration for their use in soy-based foods and supplements for cancer prevention.

Glyceollin I is the Antiestrogenic Component of Mixture

Taken together these findings implicated glyceollins, which consist of a mixture of three isomers, as novel phytochemicals exhibiting marked antiestrogenicity. However, until recently the active component of the mixture had not been identified. Most of the early research with glyceollins utilized a mixture of glyceollins I (68%), II (21%) and III (11%), however recently the individual glyceollin isomers were isolated and we performed a series of sensitive and specific biological assays to examine the activity of the individual isomers (28). Early in vivo studies demonstrated that the glyceollin mixture inhibited the estrogen-stimulated tumor growth suggesting that it may be useful in the treatment of breast cancer. The effects of glyceollin on the gene expression of three estrogen responsive genes: ER α , PgR and stromal derived factor-1 (SDF-1) were measured in MCF-7 cells. While estrogen treatment caused an increase in the expression of PgR and SDF-1, glyceollin reduced the estrogen response. In addition in vitro target gene arrays were performed in tamoxifen and glyceollin-treated MCF-7 cells on an extensive panel of genes commonly altered in breast cancer and estrogen signaling. Results demonstrated one particular gene, nerve growth factor receptor (NGFR) that was uniquely induced by glyceollin. When the individual isomers were isolated, glyceollin I was the only isomer to demonstrate a dose dependent decrease in ER transcriptional activity. In contrast glyceollin isomers II and III showed no significant inhibition of ERE potentiation, suggesting that glyceollin I is the active component of the three isomers in the mixture.

The ER is thought to mediate the mitogenic actions of estrogen and since previous studies

demonstrated the glyceollin mixture to exert antagonist activity on ER α and ER β we were then interested in evaluating the differences in binding affinity between the three glyceollin isomers. Among the three individual isomers tested, the glyceollin I isomer displayed the highest affinity for ER α with an IC₅₀ value of 1.68 μ M. The IC₅₀ values of glyceollin II and III were 6.57 μ M and greater than 10 μ M, respectively. This indicated that the ability of glyceollin I to act as an ER antagonist occurred through ER binding. Once establishing the binding affinity of the glyceollins for the ER we evaluated the ligand-receptor docking of the glyceollins to the tamoxifen induced antagonist form of the ER. Our results demonstrated that glyceollin I interacts more like tamoxifen with ER while glyceollins II and III bind in a completely different conformation. Figure 2 depicts the docking pose of glyceollin I to ER α in relation to tamoxifen which suggests how glyceollin I exerts antagonist properties. Also shown is the completely different docking poses for the glyceollin isomers II and III. In this figure, a Connolly Channel, colored green, representing the surface of the binding cavity of the ER is depicted with the three essential amino acids within the binding cavity: Arginine 394 and Glutamate 353 on the right, and Histidine 524 on the left. The experimentally defined binding pose of tamoxifen is shown in yellow while the binding pose of glyceollin I suggested by docking is shown in atom colors. The docked binding pose of glyceollin I utilizes the same phenolic hydrogen bond interactions to Arginine 394 and Glutamate 353 that are found in the actual 4-hydroxytamoxifen binding within the ER. At the same time, the glyceollin I pose has no interactions with Histidine 524 as is observed in the binding of most estrogen agonists (17 β -estradiol, DES). In panel B, the “best” fit poses for glyceollins II and III are shown in magenta and red respectively to contrast with the atom colored docking pose of glyceollin I. The best docking poses of glyceollin II and III cannot interact with Arginine 394 and Glutamate 353 or Histidine 524 and thus take on only a weak and superficial steric interaction within the binding channel induced by the tamoxifen side chain. Glyceollin II and III docking poses are also in the opposite orientation when compared with glyceollin I and the phenolic group of these glyceollins can not fit inside the ER ligand binding cavity to stabilize a binding interaction. The more linear overall ring linkage in the glyceollin I structure (Figure 2) is the primary contributing feature of its unique ability to fit well into the tamoxifen induced antagonist form of the ER.

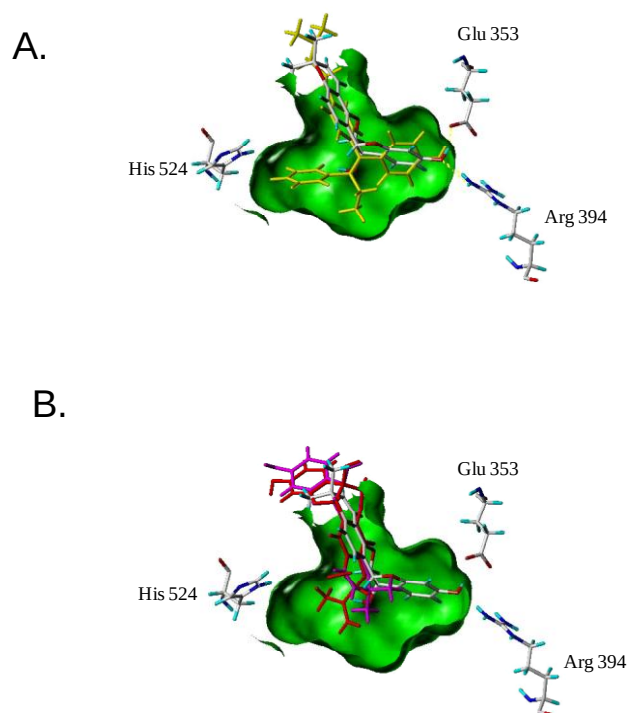


Figure 2. A and B. 4-Hydroxytamoxifen and Glyceollin I in the binding cavity of the ligand binding domain of ER α interacting with Histidine 524, Arginine 394, Glutamate 353. (A) Glyceollin I (atom colored), Glyceollin II (magenta) and Glyceollin III (red) in the binding cavity of the LBD of the ER α interacting with Histidine 524, Arginine 394 and Glutamate 353 (B).

We performed additional gene expression analyses and results demonstrated that glyceollin I inhibited estrogen-stimulated SDF-1 and PgR expression in two ER positive cell lines, MCF-7 human breast cancer cells and BG-1 ovarian cancer cells. To further test the hypothesis the glyceollin I isomer is the active component of the glyceollin mixture proliferation and viability studies were performed and results demonstrated that glyceollin I inhibited the proliferation in both cell lines.

Natural (-) Glyceollin I the Antiestrogenic Enantiomer

To further investigate the antiestrogenic properties of glyceollin I, the natural (-)-enantiomer was synthesized and the unnatural (+)-enantiomer and racemate were prepared (29). Since numerous studies with ER ligands have demonstrated enantiomer specific biological differences, (30, 31, 32) we investigated the biological differences between the glyceollin enantiomers in human breast cancer cells (33). Results demonstrated the two enantiomers displayed similar binding affinities for ER α and ER β . However, we found differential ERE

transcriptional activities between the enantiomers: (+)-glyceollin I increased the activity, while (-)-glyceollin I was inhibitory. In addition, the enantiomers displayed differential gene expression on a panel of breast cancer and estrogen receptor signaling genes. This suggested that although glyceollin I is the active isomer responsible for its antiestrogenic properties, (-)-glyceollin I is the predominate enantiomer involved in conferring inhibition.

Discussion

In summary, our data show that the soy glyceollins display unique in vitro and in vivo antiestrogenic activity. We identified glyceollin I as the active component of the combined glyceollin mixture. Further results using glyceollin I demonstrate a novel inhibition of ER-mediated gene expression and cell proliferation/survival. Synthesis of glyceollin I produced both the natural (-)-glyceollin and unnatural (+)-glyceollin I enantiomers. Although both enantiomers demonstrated similar binding affinities to ER, (+)-glyceollin I stimulated ERE-transcriptional activity and exhibited no antiestrogenic activity. Glyceollin I may represent an important component of a phytoalexin-enriched food (activated) diet in terms of chemoprevention as well as a novel therapeutic agent for hormone dependent tumors.

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Conflicts of Interest

No potential conflicts of interest to disclose.

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