Introduction

Over the past 50 years dietary estrogens have played an important role in human health and animal agriculture. In the late 1940s compounds present in subterranean clover were shown to alter reproduction in sheep. This reproductive failure had a severe economic impact on the sheep industry in Australia. However the compound responsible for the reproductive failure was not identified for over a decade. In 1953, genistein, a compound present in legumes, was shown to enhance uterine weight in rodents and, thus, was classified as a phytoestrogen (plant estrogen). In the mid-1960s several phytoestrogens were discovered because of their importance in reducing fertility in sheep grazing on subterranean clover in Australia. Not only did these sheep have fertility problems, virgin ewes and wethers expressed milk, which also is indicative of consumption of potent estrogenic compounds. In the 1970s phytoestrogens were classified as a naturally occurring toxicant in food by the National Academy of Science. It is interesting to note that in the 1990s many of the bioactive components in foods, which were previously listed as toxicants in food, are being promoted for their potential health benefits.
benefits. The phytoestrogens, specifically the soy estrogenic isoflavone genistein, has been one of the most studied phytochemicals in food over the past 5 years. It is important to note that estrogen-like compounds in food have the potential to prevent or alter many chronic diseases such as cardiovascular disease, cancer, and osteoporosis.

Estradiol, the female hormone, plays a critical role in function of the reproductive system. Additionally, estrogens are critical factors in such biological processes as cellular development, proliferation, and differentiation. Although estrogens are essential to reproductive function, it is believed that estrogens play a critical role in development of several human, hormone-dependent cancers, such as breast and uterine cancer. The biological actions of estrogen, as well as that of estrogen agonists, are mediated by a soluble protein, the estrogen receptor (ER), to which estrogen binds with high affinity. Once the ligand binds to the ER, the liganded complex undergoes transformation and a chaperone protein (Heat Shock Protein 90) dissociates. This dissociation exposes the DNA-binding domain and allows the liganded ER to form a homodimer. These homodimers bind with high affinity to specific DNA sequences, known as estrogen responsive enhancers (ERE), which are upstream of estrogen responsive genes. The liganded receptor complex bound to the ERE initiates transcription of estrogen responsive genes. Estrogen-like compounds that bind to the ER and stimulate an estrogenic response are considered estrogen agonists. Those chemicals that bind to the ER and block the estrogenic response are considered estrogen antagonists. Another class are those compounds that do not bind to the ER, but inhibit an estrogenic response such as estrogen-dependent gene expression or cell growth. It is likely that these compounds mediate their effect post-receptor binding but pre-transcriptionally. Since these compounds do not mediate the effects by blocking estrogen binding to the ER, these compounds are considered antiestrogens. The emphasis of this chapter will be on the biological activities of phytoestrogens which are found in foods and feedstuffs consumed by humans, livestock, and wildlife. We will discuss phytoestrogens that can act as estrogen agonists and antiestrogens. Collectively these estrogen-like compounds will be referred to as dietary estrogens and antiestrogens.

Affinity of estradiol (E) for the ER is high, with $K_D$ of ~ 0.1 nm. There are numerous dietary E ligands, all of which have chemical structures containing opposing hydroxyls on phenolic rings (Figure 2.1). These dietary ER ligands have affinity for the ER that are 100 to 1000 times lower than estradiol.

 Humans and animals are exposed to environmental estrogens that give rise to varying biological effects depending on the dosage and the specific chemical. Sources of these estrogens include estrogenic drugs and industrial compounds, such as pesticides, nonionic surfactants, and chemical precursors used in the manufacturing of plastics. A subclass of the environmental estrogens are the dietary estrogens, which have been identified in several plants. These compounds are classified as phytoestrogens.

Dietary estrogens comprise a diverse group of compounds with varied chemical structure and biological activities. In this chapter we will focus on
several dietary estrogens and antiestrogens. These include lignans, zearalenone, coumestans, isoflavones, and indole-3-carbinol.

**Dietary Estrogens and Antiestrogens**

**Lignans**

Lignans are found in many plant foods and make up a large portion of the known dietary phytoestrogens. Precursor compounds that form mammalian
Lignans have been identified in grains, seeds, berries, and nuts. In the case of grains, these compounds are located in the outer fiber-containing region called the aleurone layer. The first mammalian lignans were identified in vervet monkeys and human females as unknown, cyclically occurring compounds. Later, independent of each other, two groups identified these compounds as enterolactone and enterodiol. These two lignans have since become the most well known and researched compounds of their type.

The metabolism of mammalian lignans is thought to be dependent on the activity of the animal’s gut microflora. Precursor compounds from plant sources enter the digestive system of the mammal, where in the lower gut, host microbes metabolize these precursor compounds into their respective lignans. Matairesinol and secoisolariciresinol are metabolized in the lower gut into enterolactone and enterodiol, respectively. Setchell et al. and Adlercreutz proved the dependency of this metabolic pathway and microbe involvement by detecting a significant decrease in urine enterolactone and enterodiol concentration when they disrupted the normal gut flora with antibiotics. In many cases there was almost complete elimination of these compounds upon disruption of the gut flora.

The biological effects lignans have in mammals are very similar to those of the other dietary estrogens. Also, as with the other compounds discussed in this chapter, lignans carry out their function by acting as weak estrogens. Sathyamoorthy et al. demonstrated that enterolactone stimulated estrogen responsive, MCF-7 breast cancer cells to produce pS2. This result is a clear indication of the estrogenic activity of these compounds. Also, lignans are believed to have numerous other biological effects including: anticarcinogenic, antiviral, bacteriostatic, and fungistatic activities. The relationship of mammalian lignans to several different forms of cancer has been well researched in recent years. Hirano et al. demonstrated that lignans suppress mitogen-induced proliferation of human peripheral blood lymphocytes. It also has been suggested that lignans may play a role in decreasing the incidence of breast cancer by competing with estradiol for type II estrogen-binding sites and by affecting uptake and metabolism of sex hormones through regulation of synthesis of plasma sex hormone-binding globulin in the liver.

Zearalenone

Another class of dietary estrogens that occur in foodstuffs are derivatives of resorcylic acid lactones which are produced by numerous species of Fusarium fungi growing, under favorable conditions, on grains prior to and after harvest. The most prevalent of these compounds is zearalenone, 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid lactone, and its derivatives. This estrogen-like compound was first isolated from corn infected with Fusarium. Since that time, over 300 derivatives of zearalenone have been isolated. Fusarium fungi infect numerous agriculturally important crops, including cereal grains that make up a significant part of the human diet. The
grains shown to be most affected by this mycotoxin include corn, wheat, barley, sorghum, and hay. Numerous Fusarium fungi produce this mycotoxin including Fusarium roseum and F. moniliforme which invade kernels of corn and F. saubinetti that is known to infect barley. High concentrations of zearalenone are found in grain products most often as a result of an infected grain being stored, allowing the fungi to thrive. However, small amounts of zearalenone have been identified in fresh cut grains.

Zearalenone was first characterized as having estrogenic activity by Mirocha et al. when the compound was shown to increase uterine weight in rats. It was later discovered, that as is the case with many of the other dietary estrogens, zearalenone acts via the estrogen receptor. In 1978, Boyd and Wittliff performed competitive binding assays proving that zearalenone does, in fact, bind to the estrogen receptor. Since that time, binding assays have been performed with many of the derivatives of zearalenone and one derivative (low melting point zearalenol) has been identified as having the highest affinity for the estrogen receptor of any of the known dietary estrogens. These in vitro studies also concluded that, like the other dietary estrogens examined, zearalenone caused growth of estrogen-dependent MCF-7 human breast cancer cells. More recent studies have proved that zearalenone has detrimental effects on the reproductive efficiency of swine and mink. In these studies, mink receiving zearalenone mated, but only 25% whelped. In swine, hyperestrogenism generally appears when corn is contaminated with zearalenone at 1 ppm, but it can occur at doses as low as 0.1 ppm. Zearalenone has proved to be a potent environmental estrogen and as a result it is used in beef cattle production as a growth-promoting supplement.

**Coumestans**

Another class of dietary estrogens is the coumestans. These phytoestrogens are found in many vegetables and forages including soybeans, alfalfa sprouts, large lima beans, mung bean sprouts, round split peas, red bean seeds, and clover sprouts. Coumestrol is the most commonly studied coumestan and it is the predominant form found in alfalfa and other forages. However, the highest concentration of coumestrol has been measured in soybeans.

As with the other dietary estrogens, coumestrol exhibits estrogenic activity through interaction with the estrogen receptor. Coumestrol has been shown to have other biological activities related to lipid and calcium metabolism. In studies performed by Dodge et al., coumestrol, genistein, and zeranol were all shown to lower serum cholesterol in ovariectomized rats. Furthermore, coumestrol and zeranol prevented ovariectomy-induced bone loss. In a similar study, Draper et al. went on to demonstrate that coumestrol reduced urine calcium excretion and bone resorption markers pyridinoline and deoxypyridinoline after one week of treatment.
Genistein

The phytoestrogen genistein is an isoflavone with low affinity to ER, which is present in high concentrations (1 to 2 mg/g) in soybeans and soy products. Genistein is known to reduce reproductive performance of sheep grazing on subterranean clover, rabbits fed soybean hay, captive cheetahs fed diets containing soybean protein, and desert quail feeding on desert brush.\textsuperscript{53,54} All of these diets consumed by the various species contained substantial amounts of genistein. Additionally, a decrease in reproductive performance also was observed in female rats fed either a soy-based or a genistein-supplemented diet.\textsuperscript{55} Estrogenic activity from components in these diets may prevent normal estrus in these animals and is a likely mechanism by which these diets alter reproduction. Human diets, containing 60 g/d of soy products (providing 45 mg/d of isoflavones) increased the length of menstrual cycles in women,\textsuperscript{56} suggesting that dietary phytoestrogens also are capable of producing a biological response in humans.

Genistein and other isoflavones exist in plants as the glycoside conjugates. In fact, studies in the 1970s revealed that 99% of the isoflavonoid compounds in soy are present as glycosides.\textsuperscript{57} It is generally accepted that these dietary glycosides must be hydrolyzed to aglycones by gut microbiota before absorption can occur. Individuals consuming soy milk, in three different amounts, had a dose-dependent increase in plasma genistein concentration ranging from 0.74 to 2.15 \(\mu m\).\textsuperscript{58} In another study, humans weighing 61.9 kg consumed soy drinks that provided isoflavones at 30.9 \(\mu mol/kg \) body weight. This is a very high dose that provides approximately 500 mg isoflavones per day. Blood concentrations of genistein and daidzein in the individuals consuming these large amounts of isoflavones were approximately 6 \(\mu m\) each.\textsuperscript{59} Soy protein (60 g) containing 45 mg of isoflavones (20 mg genistein) given daily to women for 1 month significantly increased follicular phase length, delayed menstruation, or both.\textsuperscript{56} These results indicate that dietary soy is estrogenic in adult women.

Genistein binds to the ER with an affinity 50 to 1000 times less than that of estradiol.\textsuperscript{21} We conducted competitive-binding experiments with rat uterine cytosol and confirmed that genistein binds to the ER with an affinity 1/50 to 1/100 that of estradiol.\textsuperscript{60} As with several of the other dietary estrogens presented here, there are numerous reports that genistein can act as an agonist in ovariectomized animals as indicated by increases in uterine weight and mammary development.\textsuperscript{16} Maturation of the mammary gland was observed in pubertal Sprague-Dawley rats administered subcutaneous genistein at 500 \(\mu g/g \) body weight.\textsuperscript{61} These authors hypothesized that an ER-mediated mechanism promoted mammary epithelial cell proliferation and enhanced mammary gland maturation. In studies using ovariectomized female rats, dietary genistein at 750 ppm can enhance mammary gland development.\textsuperscript{60} Feeding genistein or estradiol to ovariectomized rats led to an increase in serum prolactin levels,\textsuperscript{60} which also suggests estrogenic action of genistein on the hypo-
thalamus and pituitary gland in vivo. Dietary genistein induced expression of the estrogen-responsive gene c-fos in uterine RNA isolated from ovariectomized rats. Further, genistein can act as an estrogen agonist to stimulate growth of cultured human breast cancer (MCF-7) cells at concentrations as low as 200 nm.\textsuperscript{13,62}

The estrogenic and antiproliferative activities of genistein present an apparent paradox. Epidemiological data suggest that diets rich in soy products, which contain high levels of phytoestrogens, are associated with a lower incidence of breast cancer.\textsuperscript{63,64} There also are numerous reports that genistein inhibits growth of cultured human cancer cells.\textsuperscript{65-67} We conducted experiments to resolve this apparent paradox by using both ER-negative (MDA-MB-231) and ER-positive (MCF-7) human breast cancer cells to evaluate the growth-inhibitory effect of genistein on cultured breast cancer cells. At concentrations above 20 μm, we observed a dose-dependent decrease in growth of both MDA-MB-231 and MCF-7 cells.\textsuperscript{68} This inhibitory effect is independent of ER because it is observed in both ER-positive and ER-negative cells.

Blood concentrations of genistein reported in humans consuming soy-containing diets are relatively low. To determine whether lower levels of genistein could induce an estrogenic response \textit{in vitro} in ER-positive cells, we\textsuperscript{64} and others\textsuperscript{69} conducted dose-response studies in MCF-7 cells in which the concentration of genistein (in charcoal-stripped media) ranged from 10 nm to 100 μm. Results from these studies support the dual threshold hypothesis: when genistein is administered at low concentrations, a dose-dependent increase in MCF-7 cell growth is observed, with maximal growth occurring at 1 μm, whereas concentrations above 20 μm lead to a dose-dependent inhibition of growth. Genistein concentrations of 1 μm and 10 μm were also evaluated in MCF-7 cells by determining changes in expression of \textit{pS2} mRNA by Northern blot analysis.\textsuperscript{64,70,71} Expression of \textit{pS2} is an established marker of estrogen-dependent gene expression.\textsuperscript{71}

**Indole-3-Carbinol and Metabolites as Antiestrogens**

Indole-3-carbinol (I3C) is a secondary plant metabolite found in cruciferous vegetables, such as cabbage, Brussels sprouts, and broccoli. Consumption of these vegetables has been associated with decreased risk for cancer in humans.\textsuperscript{72} I3C has been evaluated in human clinical trials as a potential chemopreventive agent against breast and ovarian cancers.\textsuperscript{73} It has been known that I3C suppresses the growth of both estrogen-dependent and estrogen-independent human breast cancer cell lines.\textsuperscript{74} Dietary I3C has been reported as inhibiting spontaneous tumorigenesis and tumor induction by direct-acting carcinogens\textsuperscript{74-78} in various estrogen-responsive target organs, including mammary tissue,\textsuperscript{79-81} liver,\textsuperscript{82,83} endometrium,\textsuperscript{84} lung,\textsuperscript{85-88} and other target organs\textsuperscript{89,90} in various animal models. However, there are other studies that demonstrated stimulation of tumor promotion by I3C \textit{in vivo}.\textsuperscript{91,92}
I3C was administered orally to animals, it induced chemopreventive effects against a wide variety of carcinogens. Chemoprevention properties of dietary I3C in most of the models are evident when it is administered with the carcinogens or prior to initiation. There are reports that, when given after initiation (promotion-progression stage), I3C can enhance carcinogenesis.\textsuperscript{75,77,83} There also is some evidence that I3C may be mutagenic when administered in the diet along with nitrites.\textsuperscript{95} Earlier studies\textsuperscript{77,83} documented the ability of I3C to promote aflatoxin B$_1$-initiated hepatocarcinogenesis at relatively high dietary levels (1000 ppm).

The chemopreventive properties of I3C are proposed to occur through several possible mechanisms, including the alteration of estrogen metabolism.\textsuperscript{81,96-99} I3C is known to inhibit glutathione S-transferase-mediated steroid binding activity,\textsuperscript{100} act as a scavenger of free radicals,\textsuperscript{101} modulate the activity of multidrug resistance,\textsuperscript{102} and alter the expression of various phase I and II drug metabolizing enzymes\textsuperscript{99,103-106} contributing to detoxification of carcinogenic compounds. Dietary intake of I3C has antiestrogenic as well as estrogenic activities\textsuperscript{107} and also binds to the arylhydrocarbon receptor (AhR).\textsuperscript{99,108,109} I3C is known to be an inducer of intestinal and hepatic xenobiotic metabolizing enzyme activities.\textsuperscript{105,110-112} Although I3C induces several phase II enzymes,\textsuperscript{112} the indoles induce multiple families of cytochrome P450-dependent isozymes. I3C induces CYP1A family (e.g., TCDD), CYP2B family (e.g., phenobarbital), and CYP2A family (e.g., dexamethasone) isozymes.\textsuperscript{79,103,106} Grubbs et al.\textsuperscript{79} report that after 15 weeks of exposure to I3C, the livers of Sprague-Dawley rats continue to have higher activities of both phase I and II enzymes. I3C acts as an antinitiator as well as a promoter of carcinogenesis, and increases in activities of cytochrome P450-dependent monoxygenases and in phase II enzymes (conjugation).\textsuperscript{109} Many of the aromatic hydrocarbon receptor (AhR) agonists are environmental toxicants.\textsuperscript{113} These researchers found that I3C was an AhR agonist with weak binding affinity and an inducer of monoxygenase activity in vivo. It has been reported that I3C binds to the same AhR site as a potent environmental pollutant, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and other arylhydrocarbons.\textsuperscript{108} I3C exhibited antiestrogenic activities at concentrations that did not induce ethoxyresorufin O-deethylase activity (EROD). An additional mechanism for the chemopreventive effects of I3C in estrogen-responsive tissues is a modulation of cytochrome P450-dependent estradiol metabolism. Estradiol is metabolized via two competing pathways. Hydroxylation at C-2 yields 2-hydroxyestrone; hydroxylation at C-16\textalpha yields 16\textalpha-hydroxyestrone which is reduced to form estriol.\textsuperscript{114-116} 16\textalpha-hydroxyestrone covalently binds to the estrogen receptor, decreases its degradation and has estrogenic effects. Increased estradiol-16\textalpha-hydroxyestrone has been associated with increased risk for breast cancer in women\textsuperscript{114} and mice,\textsuperscript{116} and 16\textalpha-hydroxyestrone has been reported to be genotoxic in mammary cells.\textsuperscript{117} Attempts to decrease estradiol 16-hydroxylation have not been successful. Thus, their studies focused on increasing
the alternate 2-hydroxylation pathway of estradiol.\textsuperscript{96,97} In rodents, I3C induces CYP1A1/CYP1A2-dependent estradiol 2-hydroxylase activity and the formation of 2-hydroxyestradiol/2-hydroxyestrone has been associated with protection from estrogen-induced mammary, endometrial, and other tumors development.\textsuperscript{81,84,116,118-121} Toshifuma et al.,\textsuperscript{121} reported that I3C increased 2-hydroxylation in estrogen-dependent human breast cancer cells but has little effect on 16a-hydroxylation. In human breast cancer cells, induction of estradiol 2-hydroxylase activity is a CYP1A1-dependent response, and several studies have reported induction of this activity by AhR agonists, I3C.\textsuperscript{81,122,123} However, I3C induced CYP1A1 in MCF-7 cells only at high concentrations (500 mM),\textsuperscript{122} and induced CYP1A1 mRNA only at concentrations \( \geq 100 \) mM.\textsuperscript{123} In contrast, McDougal et al.\textsuperscript{124} reported that after 48 h incubation of MCF-7 cells with 10 mM, I3C resulted in a more than fourfold increase of estradiol 2-hydroxylase activity.\textsuperscript{125} Therefore, induction of estradiol 2-hydroxylase by 10 mM, I3C may be CYP1A1-independent or may involve \textit{in vitro} activation of P450 isoenzymes in MCF-7 cells.

Many of these enzyme-inducing effects are due to the condensation products of I3C produced upon contact with gastric acid.\textsuperscript{104,109,126} Some of these oligomers have been shown to interact with the AhR. This may be involved in induction of the CYP1A family which is thought to be primarily responsible for the inactivation of estradiol in breast tumor cells and other drug metabolizing enzymes. Increased estrogen conjugation and excretion via induction of phase II enzymes could result in these effects.\textsuperscript{127} A major condensation product, the dimer 3,3'‐diinloylmethane is an effective inhibitor \textit{in vitro} of cytochrome P450.\textsuperscript{109,128,129} Oligomers of I3C enhance estradiol 2-hydroxylation in the human through the CYP1A family.\textsuperscript{97} Indolo[3,2-b]carbazole (ICZ) is one of the acid-condensation products of I3C that is produced \textit{in vivo} and \textit{in vitro}.\textsuperscript{109} ICZ binds to both the estrogen receptor and AhR. ICZ decreases estrogen receptor levels in breast cancer cells in culture.\textsuperscript{107} ICZ competitively binds to the AhR, induces P450 (CYP1A1/2) gene expression, and transforms the cytosolic AhR to a form that binds to a dioxin or xenobiotic responsive element.\textsuperscript{99,108,109,130,131} ICZ is the most potent AhR agonist among condensation products of I3C. Like I3C, ICZ is also similar to the TCDD. Both compounds exhibit antiestrogenic activities including inhibition of estrogen-dependent growth of cultured breast tumor cells.\textsuperscript{107} And, both substances induce CYP1A1 activity \textit{in vivo} and \textit{in vitro}.\textsuperscript{109} ICZ is not only an inducer of the CYP1A1 gene, but also a potent and selective inhibitor of CYP1A1 enzyme activity.\textsuperscript{132} ICZ inhibited estradiol-induced cell proliferation at concentrations above 10 nm.\textsuperscript{132} At lower dietary I3C levels (<1000 ppm), estrogenic activities of I3C acid derivatives promote hepatocarcinogenesis in rainbow trout. Much stronger promotion was induced at high dietary I3C levels (≥1000 ppm), at which levels of CYP1A also were induced.\textsuperscript{133} I3C and related condensation products also have been characterized as AhR agonists and exhibit structure-dependent binding affinity for the AhR.\textsuperscript{108,109,131}
Summary

The dietary estrogens and antiestrogens discussed in this chapter are found in fairly high concentrations in many foods that are routinely consumed daily. For example, lignans are found in foods that are high in fiber. Genistein is found in soy foods. Zearalenone is found in moldy corn which has been contaminated with Fusarium. Many of the dietary estrogens are present in high concentrations; for example, genistein has been found in foods ranging from 0.8 to 1.2 mg/g of food.

It is important to note regarding the dietary estrogens that although these chemicals bind weakly to the ER, they are in high concentrations in foods. For example, genistein has a low affinity relative to estradiol for binding to the ER. However, genistein is in concentrations of 1 mg/g of soy food (dry matter). Thus, it is possible for an individual to easily consume 50 mg in one day. This dietary consumption of genistein would produce a circulating plasma concentration in excess of 200 nm (aglucone form). This plasma concentration is 200 times higher than the concentration of estradiol in a premenopausal woman. Even though genistein is a weak agonist, the concentration is high enough to elicit an estrogenic response. Regarding the dietary antiestrogens, many of the chemicals discussed are found in Brassica vegetables, such as cabbage, Brussel sprouts, and broccoli.

In the past decade, many of the estrogens and antiestrogens discussed in this chapter have been shown to be protective against several types of cancers. Because of the potential beneficial effects of these chemicals, extracts containing high concentrations of these bioactive chemicals are now available as dietary supplements or for use as food additives. Additionally, plant geneticists are now selecting cultivars which are high in some of these chemicals. For example, soybeans containing high amounts of isoflavones are currently under investigation. Additionally, broccoli cultivars with high concentration of glucobrassicans also are available. At some point, the content of these chemicals will become too high and become a chemical safety concern. Currently it is believed that consumption of levels of these bioactive chemicals is safe as long as the levels do not exceed that found in food. If we increase the content of these chemicals by selection or genetic engineering to five times the average levels, can we still assume this is safe? Another important point that must be made is regarding subpopulations. If we develop a food with high concentrations of isoflavones as a “natural” mechanism to consume estrogen-like chemicals for prevention of bone mineral loss, is this same product safe for another subpopulation with an estrogen-dependent cancer? The safety of consumption of high amounts of dietary estrogens and antiestrogens remains an important unanswered question.
References


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