Nutrition and Cancer

Brown Kelp Modulates Endocrine Hormones in Female Sprague-Dawley Rats and in Human Luteinized Granulosa Cells[1]

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ABSTRACT Epidemiological studies suggest that populations consuming typical Asian diets have a lower incidence of hormone-dependent cancers than populations consuming Western diets. These dietary differences have been mainly attributed to higher soy intakes among Asians. However, studies from our laboratory suggest that the anti-estrogenic effects of dietary kelp also may contribute to these reduced cancer rates. As a follow-up to previous findings of endocrine modulation related to kelp ingestion in a pilot study of premenopausal women, we investigated the endocrine modulating effects of kelp (*Fucus vesiculosus*) in female rats and human luteinized granulosa cells (hLGC). Kelp administration lengthened the rat estrous cycle from 4.3 ± 0.96 to 5.4 ± 1.7 d at 175 mg · kg^{-1} body wt · d^{-1} ($P = 0.05$) and to 5.9 ± 1.9 d at 350 mg · kg^{-1} · d^{-1} ($P = 0.002$) and also led to a 100% increase in the length of diestrus ($P = 0.02$). Following 175 mg · kg^{-1} · d^{-1} treatment for 2 wk, serum 17β-estradiol levels were reduced from 48.9 ± 4.5 to 40.2 ± 3.2 ng/L ($P = 0.13$). After 4 wk, 17β-estradiol levels were reduced to 36.7 ± 2.2 ng/L ($P = 0.02$). In hLGC, 25, 50, and 75 μmol/L treatment reduced 17β-estradiol levels from 4732 ± 591 to 3632 ± 758, 3313 ± 373, and 3060 ± 538 ng/L, respectively. Kelp treatment also led to modest elevations in hLGC culture progesterone levels. Kelp extract inhibited the binding of estradiol to estrogen receptor α and β and that of progesterone to the progesterone receptor, with IC_{50} values of 42.4, 31.8, and 40.7 μmol/L, respectively. These data show endocrine modulating effects of kelp at relevant doses and suggest that dietary kelp may contribute to the lower incidence of hormone-dependent cancers among the Japanese. J. Nutr. 135: 296–300, 2005.

KEY WORDS: • *Fucus vesiculosus* • *rat* • *seaweed* • *breast cancer* • *estrogen*

The rise in estrogen-dependent cancers in the United States and our limited success with their prevention and treatment have spurred growing interest in the dietary habits of the Japanese, who have one of the lowest rates of breast, endometrial, and ovarian cancers in the world (1,2). Studies show that Japanese women have longer menstrual cycles and lower serum estradiol levels than their Western counterparts (3–5), factors that may contribute to their low risk of estrogen-dependent cancers. To date, these low rates have been partly attributed to the soy-rich diets inherent among Asian populations (6–8). However, another contributory factor may be their high intake of seaweed, as previously hypothesized by Teas et al. (9). In a human pilot study, we demonstrated that intake of the brown kelp seaweed, *Fucus vesiculosus* (bladderwrack), significantly increased the total number of days of the menstrual cycle, reduced circulating 17β-estradiol levels, and elevated serum progesterone levels in premenopausal women with abnormal menstrual cycling histories (10). In the present study, we have further investigated the endocrine modulating effects of *F. vesiculosus* on sex hormone levels and cycling patterns in rats and in a human model.

Previous studies show an inverse relation between menstrual cycle length and risk of breast (11), ovarian (12), and endometrial (13) cancers. Menstrual cycle length and age of onset of menarche and menopause may serve as surrogate measures of endogenous estradiol and progesterone exposure [reviewed in (14)]. Women with shorter cycles experience a greater total number of menstrual cycles during the course of their reproductive lifetimes than those with longer cycling patterns. Hence, these women will spend more time overall in the follicular and luteal phases of the cycle where estrogen and progesterone levels and endometrial and breast cell proliferation rates are at their highest. Positive associations also have been reported among breast, endometria, and ovarian cancers and obesity and alcohol intake, factors that promote persistent estrogenic stimulation and hormone imbalances (15–17). These studies suggest that exposure to estrogens and an imbalance in the estrogen/progesterone ratio may be the most critical determinants in risk of estrogen-dependent diseases. Inhibition of estrogen via the estrogen receptor or aromatase

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blockade is a current strategy for prevention in high-risk individuals and in the treatment of some estrogen-dependent diseases. Furthermore, the identification of dietary components that exert chemoprotective effects by suppressing endogenous estrogen production may provide another means to reduce the incidence of breast, endometrial, and ovarian cancers.

Rat and primary human luteinized granulosa cell (hLGC)[4] models have been utilized to examine chemical endocrine disruptor effects on disease risk. The Sprague-Dawley female rat has been used as a model to investigate the effects of endocrine modulation on mammary, ovarian, and endometrial carcinogenesis (18–20). Chemical endocrine disruptors are commonly tested using the rat model due to similarities in ovarian hormone responsiveness between humans and rats (21,22). In both the rat estrous cycle and the human menstrual cycle, estradiol levels peak during proestrus and the follicular phase and progesterone levels peak during diestrus and the luteal phase, respectively. Primary hLGC cultures simulate granulosa lutein cells of the corpus luteum in vivo because they support the timely and dynamic secretion of estradiol and progesterone in patterns that mimic serum hormone levels during the luteal phase of the menstrual cycle. Thus hLGC cultures have been utilized successfully in the study of chemical endocrine disruption, such as the effects of dioxin on ovarian estrogen and progesterone synthesis (22).

Our objectives in the present study were to build upon our previous findings using these models by *1*) examining whether dietary administration of *F. vesiculosus* disrupts normal estrous cycling and sex steroid secretion in Sprague-Dawley rats and *2*) treating hLGC cultures with a *F. vesiculosus* extract to study differences in endocrine responses in granulosa cells. To further investigate possible mechanisms of action of the *F. vesiculosus* extract in estrogen and progesterone responses, we evaluated its binding affinity to estrogen receptor (ER)α, ERβ, and progesterone receptor (PR)-B and its potential to inhibit aromatase activity in hLGC cultures.

MATERIALS AND METHODS

Rats and estrous cycle monitoring. Twenty-four female adult Sprague-Dawley rats (Charles River Canada) weighing 200–250 g were individually housed in wire cages. They were allowed ad libitum access to a standard laboratory diet (AIN-76) (23) and water. Following a brief 2-wk adjustment period, rats underwent daily vaginal cytology monitoring to determine normal estrous cycling using staging criteria described by Everett (24). Rats were required to have at least 2 normal, consecutive estrous cycles prior to experimentation. A normal estrous cycle was defined as a 3- to 5-d cycle. A complete estrous cycle was defined as the day of estrus to the day before the subsequent estrus. Weights of rats were monitored weekly throughout the experiment.

***Source and dosing of* F. vesiculosus.** Dried, powdered *F. vesiculosus* was obtained from Maine Coast Sea Vegetables. The kelp was harvested from the Gulf of Maine and from the coastal waters of New Brunswick and Nova Scotia during the late summer months. Processing entailed sun drying the entire plant less the holdfast (root system). The dried seaweed was then milled into a fine powder.

Dosage levels were chosen to fall within the range of effective doses in our previous human studies and in a traditional Asian diet, taking into account that higher doses are often needed in rats to produce effects comparable to those seen in humans due to the increased rate of metabolism of the rat. Ethical approval was obtained for the study and all of the studies were conducted in full compliance with the U.C. Berkeley Animal Care and Use Committee guidelines.

[4] Abbreviations used: ER, estrogen receptor; hLGC, human luteinized granulosa cell; MEM, minimum essential medium; PR, progesterone receptor.

Kelp dose finding experiment (Expt. 1). Normally cycling rats were randomly divided into 3 groups of 8: a vehicle control, a low dose (175 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}), and a high dose (350 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}) group. Powdered *F. vesiculosus* was measured and applied in the morning daily to a 2-g fresh apple wedge used as a vehicle. There was a dual advantage to using an apple vehicle in this study: (1) it eliminated stress associated with gavage; (2) rats eagerly ate the apple and the kelp in this manner, making it easy to monitor and ensure complete deliverance of the kelp. Vaginal smears were obtained and daily logs were maintained to monitor estrous cycling. After ~4 wk, all rats were removed from treatment. No adverse effects were observed during the course of the experiment.

Time course experiment (Expt. 2). For serum hormone studies and to determine whether dosing over time altered hormone levels, 1-mL blood draws were taken from the tail veins of 19 normally cycling rats during the morning of proestrous (determined by vaginal cytology). Immediately following the blood draw, rats were given 175 mg $\cdot$ kg^{-1} $\cdot$ d^{-1} kelp. At 2- and 4-wk intervals, blood was redrawn during the morning of proestrus. Blood samples were allowed to clot at room temperature and were centrifuged for 10 min at 2000 $\times$ *g*. Serums were aspirated and frozen at -20°C until further analysis.

High dose experiment (Expt. 3). To determine whether high dose kelp treatments would exert anti-estrogenic and/or progestagenic effects in rats with high circulating estradiol levels, 8 rats were chosen whose estradiol levels were approximately $\geq$50 μg/L. Rats were dosed 350 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}after a baseline blood draw was taken during the morning of proestrus. Following 1 wk of treatment, an additional blood draw was taken during the morning of proestrus.

Animal hormone assays. Blood serum progesterone was assayed in triplicate using an ELISA kit (Product No. DSL-10–6800) from Diagnostic Systems Laboratories according to the manufacturer's directions. 17β-Estradiol was assayed in duplicate by a radioimmune antibody assay according to the method described previously (25).

Crude seaweed extractions. Dried, powdered *F. vesiculosus* (50 g) was placed in a 1:1 solution of distilled water and 100% ethanol, covered, and stirred for 24 h at room temperature. The extract was centrifuged for 10 min at 4500 $\times$ *g*, sterile filtered, evaporated to dryness using a rotary evaporator, and resolubilized to the desired concentrations in 50% ethanol. The estimated molecular weight of the crude seaweed extract was 300 g/mol, a value commonly used in the pharmaceutical industry for testing bioactivity of unknown plant compounds.

hLGC culture and treatment. Granulosa cells were obtained from 8 women undergoing assisted reproduction treatment at a fertility clinic. Cells were prepared, plated, and cultured as previously described for each patient (26). Briefly, cells were plated in minimum essential medium (MEM) supplemented with 0.1 IU/mL human chorionic gonadotropin, antibiotics, and 5% fetal calf serum at 37°C in an atmosphere of 5% CO_2 in air. After 48 h, cells were treated on consecutive days for 9 d with ethanol (vehicle control) or 25, 50, or 75 mmol/L kelp extract. Samples of medium from final day of treatment were assayed for 17β-estradiol and progesterone.

Granulosa cell hormone assays. Estradiol and progesterone measurements were performed using commercially available RIA kits (Diagnostic Products) as previously reported (27).

Estrogen and progesterone receptor binding assays. Affinity of the kelp extract to ERα, ERβ, and PR-B was determined by radiometric competitive binding assays as previously described (28,29) by an outside laboratory (MDS Panlabs). Briefly, dried kelp extract in 3 dilutions (0.5, 5, and 50 μmol/L final concentration) were resolubilized in dimethyl sulfoxide, combined with ERα or ERβ and 0.5 nmol/L estradiol, and mixed for 2 h at 25°C. Nonspecific binding was estimated in the presence of 1 μmol/L diethylstilbesterol. To test PR-B binding, kelp extracts were incubated for 2 h with PR-B and 1.4 nmol/L radiolabeled progesterone at 4°C. Nonspecific binding was estimated in the presence of 1 μmol/L progesterone. All determinations were carried out in triplicate and data are means $\pm$ SEM.

Aromatase activity measured using a tritiated water assay. Aromatase activity was estimated by measuring the incorporation of tritium from androstenedione into ^3H_2O as previously described (30,31). Incubations of hLGCs in 500 μL MEM with 300 nmol/L androstenedione (10% labeled, 90% radio inert, Steraloids) were

carried out at 37°C for 2 h in the presence or absence of the kelp extract (10, 50, and 100 μmol/L).

Statistical analyses. In Animal Expt. 1, differences between the means of the 3 groups were evaluated by two-way ANOVA using Proc Mixed in SAS and values are means ± SD. Dunnett's pairwise comparison procedure was used to evaluate the pairwise differences between treatments and the control group. For all other experiments, statistical analyses were performed by paired *t* tests (2-sided) with a commercially available statistical software package (Statsoft) and results were considered significant for $P < 0.05$. Values are means ± SEM.

For the radioligand-binding assay, IC_{50} values were determined by a nonlinear, least squares regression analysis using Data Analysis Toolbox (MDL Information Systems).

RESULTS

Animal studies

Kelp dose finding experiment (Animal Expt. 1). The estrous cycle was evaluated daily for ~30 d in 24 female Sprague-Dawley rats. Kelp administration led to a profound, dose-dependent increase in the length of the estrous cycle in rats fed 175 and 350 $mg \cdot kg^{-1} \cdot d^{-1}$ kelp ($P = 0.004$). In the controls, the mean number of days of the estrous cycle was 4.3 ± 0.96 compared to 5.4 ± 1.7 in the 175 $mg \cdot kg^{-1} \cdot d^{-1}$ dose group ($P = 0.05$) and 5.9 ± 1.9 d in the 350 $mg \cdot kg^{-1} \cdot d^{-1}$ dose group ($P = 0.002$). Furthermore, kelp treatment led to an overall 100% increase in the mean length of the diestrus phase of the estrous cycle ($P = 0.02$). Specifically, the mean number of days in diestrus was 0.97 ± 0.22 among the controls compared to 1.4 ± 0.54 for the 175 $mg \cdot kg^{-1} \cdot d^{-1}$ dose group and 2.1 ± 0.88 for the 350 $mg \cdot kg^{-1} \cdot d^{-1}$ dose group ($P = 0.02$). Treatment had no significant effect on the number of days in estrus, proestrus, or metestrus during the mean estrous cycle. Total number of days monitored was 28.6 ± 3.1, 30.5 ± 3.6, and 31.9 ± 3.6 for the 0, 175, and 350 $mg \cdot kg^{-1} \cdot d^{-1}$ groups, respectively.

Following Expt. 1, 5 rats stopped normal estrous cycling and were excluded from the remainder of experiments. One remained in estrus and 4 in diestrus.

Effects on serum estradiol and progesterone levels (Animal Expt. 2). Following the 175 $mg \cdot kg^{-1} \cdot d^{-1}$ treatment for 2 wk, mean serum 17β-estradiol levels were reduced from 48.9 ± 4.5 to 40.2 ± 3.2 ng/L ($P = 0.13$) and after 4 wk at the same dose levels were significantly reduced 25% from baseline to 36.7 ± 2.2 ng/L ($P = 0.02$), suggesting an effect of dosing over time. Serum progesterone levels between controls and the treatment groups did not differ.

High dose experiment (Animal Expt. 3). In the 8 rats with high circulating serum 17β-estradiol levels following 1 wk kelp administration (350 $mg \cdot kg^{-1} \cdot d^{-1}$), median serum 17β-estradiol levels decreased by 38% ($P = 0.02$) (**Fig. 1**). The range in reduction of serum 17β-estradiol levels in 6 rats was 25–58%, whereas 2 rats did not respond to kelp at all. Progesterone levels were not significantly affected following high dose treatment.

Effects of kelp treatment on17β-estradiol and progesterone levels in human granulosa cells. In hLGC cultures, the 50 and 75 μmol/L doses significantly reduced 17β-estradiol levels by 30 and 35%, respectively (**Table 1**). Kelp treatment also led to modest elevations in progesterone in hLGC medium; however, only the 50 μmol/L dose was increased ($P = 0.03$).

Radioligand binding assay. In competitive radioligand binding assays, the kelp extract exerted inhibitory effects on the binding of estradiol to ERα, ERβ, and progesterone to PR-B (**Table 2**). These data demonstrate that *F. vesiculosus* extracts compete for and bind to ERα, ERβ, and PR-B, with a slightly higher affinity for ERβ.

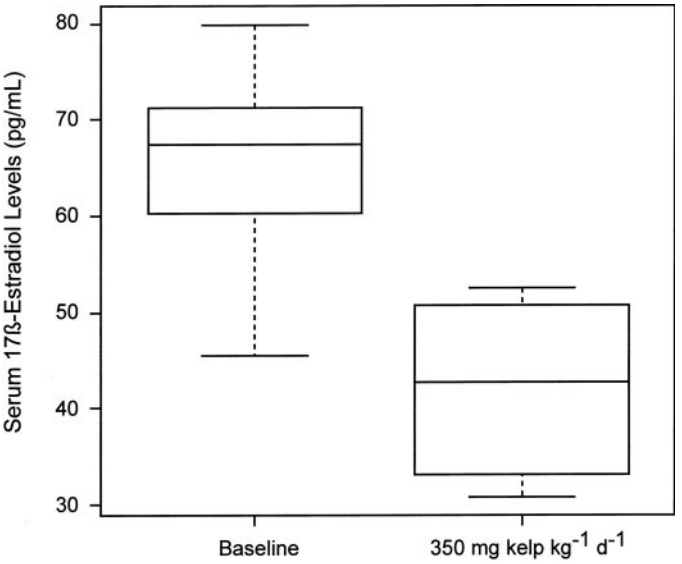

FIGURE 1 17β-Estradiol levels in rats following 1 wk of 350 $mg \cdot kg^{-1} \cdot d^{-1}$ kelp administration. The box plots indicate that in rats with high circulating serum 17β-estradiol levels median serum 17β-estradiol levels significantly decreased from 68.6 to 42.8 ng/L ($P = 0.02$); $n = 8$.

Aromatase activity following treatment of hLGCs with the kelp extract did not differ (data not shown).

DISCUSSION

Here we report additional evidence of the anti-estrogenic bioactivity of dietary *F. vesiculosus* by demonstrating its effects on rat estrous cycling patterns and serum hormone levels and on estradiol production in treated hLGC cultures. Specifically, dietary kelp resulted in an overall 37% increase in the length of the rat estrous cycle in a dose-dependent manner and led to a prolonged diestrus phase of the cycle in the 350 $mg \cdot kg^{-1} \cdot d^{-1}$ group. Kelp administration also exerted a tempering effect on estrogen production in rats, which led to 18–33% reductions in circulating 17β-estradiol levels. These findings are consistent with the observed increase in menstrual cycle length and decrease in serum estradiol levels in women following kelp administration (10). Moreover, the doses used in this study are physiologically relevant doses and are consistent with the range of intakes of 3–13 g/d estimated in Japanese populations (32). Previous studies investigating the role of dietary soy or genistein on the rat estrous cycle showed either no effects (33) or only a modest 10% increase in cycle length (34), suggesting that kelp may exert a greater effect in increasing cycle length than soy intake.

The anti-estrogenic bioactivity of *F. vesiculosus* was further demonstrated in an hLGC bioassay where dosing with kelp extract led to 23–35% reductions in 17β-estradiol levels in cell cultures. This would suggest that the extract might act by either inhibiting estradiol production or enhancing its metabolic breakdown. Competitive inhibition, altered expression, or posttranslational modification of any one of a number of cytochrome P450 enzymes involved in steroidogenesis (including cholesterol transport) or in 17β-estradiol metabolism could affect estradiol levels (22,35). However, we found no inhibitory effects of the kelp extract on aromatase activity,

TABLE 1

17β-Estradiol and progesterone levels in medium of hLGC treated with 0, 25, 50, or 75 μmol/L kelp extract[1]

Kelp dose	Estradiol	*P*-value[2]	Progesterone	*P*-value[2]
μmol/L	*ng/L*		*μg/L*	
0	4732 ± 591	—	6815 ± 1018	—
25	3632 ± 758	0.09	7721 ± 1415	0.19
50	3313 ± 373	0.03	7461 ± 923	0.03
75	3060 ± 538	0.03	7703 ± 2113	0.12

[1] Values are means ± SEM, $n = 4$.
[2] Based on *t* tests between the control and the intervention dose.

which was considered a potential molecular target due to the fact that a number of plant compounds exert highly specific inhibitory activity against this enzyme (36–38). Further studies of the mechanism by which kelp extracts inhibit estradiol production in hLGC cultures are underway.

Another potential mechanism of endocrine modulation is the competitive inhibition of hormone receptors (39). Kelp extract served as a competitive inhibitor to the binding of estradiol to both ERα and ERβ, with a slightly greater selectivity toward ERβ than ERα. These findings suggest that compounds found in *F. vesiculosus* could act as estradiol antagonists by decreasing the affinity of either ERα or ERβ for its ligand. Both receptors, which act as ligand-activated transcription factors in target genes, are found in a wide variety of tissues. Despite the relatively similar binding affinities of ERα and ERβ for estradiol, differences in binding specificity between the α and β ERs and other ligands have been observed (40), although their disparate function in target tissues remains to be fully characterized.

In the present study, there was no evidence of progestagenic effects of dietary kelp administration in Sprague-Dawley rats, and only small increases in progesterone were detected in hLGC cultures following treatment with the kelp extract. Yet, it was previously shown that *F. vesiculosus* administration corrected a progesterone deficiency in a woman with a luteal phase defect exhibiting high circulating estradiol levels (10). These discrepancies may be due to the abnormally high estradiol-to-progesterone ratio in the subject previously studied. There is evidence that estradiol, at relatively high but physiologic concentrations, is a direct inhibitor of 3β-hydroxysteroid dehydrogenase, resulting in progesterone suppression in human luteal cells (41,42). Thus, in those with high circulating estradiol, the anti-estrogenic activity of *F. vesiculosus* may not only abrogate estradiol production, but also enhance progesterone formation by alleviating estradiol's inhibitory effects on 3β-hydroxysteroid dehydrogenase. However, the potential impact of dietary kelp on circulating progesterone levels needs to be studied in a larger population.

One limitation of this study is that all rat blood was drawn during the morning of proestrus when estradiol levels were highest, but progesterone levels were not at their peak. Due to multiple blood draws from each rat, we were unable to obtain blood during other phases of the rat estrous cycle. However, we were able to determine the relative binding affinity of the kelp extract for PR-B, suggesting that kelp may act as a PR agonist.

Chemical analysis of *F. vesiculosus* has revealed that it contains many potentially bioactive compounds (43). Currently, we are in the process of identifying which agents in *F. vesiculosus* are responsible for its anti-estrogenic activity. Possible candidates include the bioactive polyphenols, sulfated polysaccharides, and the fucosterols found in several brown algae species. Polyphenols constitute ~15% of *F. vesiculosus*, with 25% of this fraction consisting of high-molecular-weight polymers. Phlorotannins, oligomers, and polymers of phloroglycinol (1,3,5-trihydroxybenzene) are the largest polyphenolic group found in *F. vesiculosus* and other brown algae. These compounds have been shown to exert bactericidal activity (44), reactive oxygen species inhibition (45), and inhibitory effects on human immunodeficiency virus type 1 reverse transcriptase and protease (46). The sulfated polysaccharides, known as fucoidans, are also found in brown seaweeds. Anti-viral (47), anti-proliferative (48), anti-angiogenic, and anti-tumor (49) properties of fucoidans have recently been described. Because of this complex chemical makeup, the precise nature of the compound(s) responsible for the endocrine-modulating effects described in this study will be difficult to elucidate but efforts are underway to isolate candidate compounds.

Our data suggest that brown seaweed intake may contribute to the lower hormone-dependent cancer rates seen in Asian populations. However, to date we have studied only the *F. vesiculosus* species that is found in North America. Whereas brown seaweed is a major constituent of the Asian diet, the primary brown seaweeds consumed among Japanese populations include wakame (*Unaria pinnatifida*) and kombu (*Laminaria japonica*) and not *F. vesiculosus*. Further studies of the potential endocrine modulating effects of these more commonly consumed seaweeds are needed along with dietary studies of seaweed intake and cancer risk using nutritional epidemiology before firm conclusions can be made.

In summary, the detection of dietary components that have estrogen-reducing effects holds promise as a simple means of dietary modification to reduce risk of estrogen-dependent cancers in the general population. Furthermore, the identification of the anti-estrogenic components in *F. vesiculosus* may lead to the discovery of novel selective estrogen receptor modulators that may be useful in the treatment and/or prevention of estrogen-dependent cancers. To this end, the isolation and identification of active components are currently in progress.

TABLE 2

Percentage inhibition, IC_{50} and K_i values of kelp extracts (0.5, 5, and 50 μmol/L) on the binding of radioligands to their respective receptors (ERα, ERβ, and PR-B) in competitive radioligand binding assays

Receptor	Kelp extract concentration[1]	% Inhibition[2]	Mean 50% inhibitory concentrations (IC_{50})	Inhibition constant (K_i)
	μmol/L		*μmol/L*	
ERα	50	52 ± 0.9	42.4	12.1
	5	21 ± 2.1		
	0.5	7 ± 2.8		
ERβ	50	58 ± 5.3	31.8	5.58
	5	18 ± 2.9		
	0.5	2 ± 2.7		
PR-B	50	55 ± 1.4	40.7	15.8
	5	12 ± 2.0		
	0.5	−0.2 ± 5.3[3]		

[1] Assayed in triplicate.
[2] Values are means ± SEM.
[3] Negative values correspond to stimulation of binding.

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