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# Black Cohosh Acts as a Mixed Competitive Ligand and Partial Agonist of the Serotonin Receptor

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Extracts of the rhizome of black cohosh [*Actaea racemosa* L., formerly called *Cimicifuga racemosa* (L.) Nutt.] were evaluated for potential mechanisms of action in the alleviation of menopausal hot flashes. Ovariectomized Sprague−Dawley rats were administered a 40% 2-propanol extract of black cohosh [4, 40, and 400 mg/(kg·day)] by gavage for 2 weeks with or without estradiol [50 $\mu$g/(kg·day)] to determine if black cohosh could act as an estrogen or antiestrogen on the basis of an increase in uterine weight or vaginal cellular cornification. No effects were observed on uterine weight or on vaginal cellular cornification in rats treated with black cohosh alone or in combination with 17$\beta$-estradiol, indicating this black cohosh extract had no estrogenic or antiestrogenic properties in the ovariectomized rat model. To evaluate other potential pathways by which black cohosh might reduce menopausal hot flashes, serotonin activity was first assessed by the inhibition of radioligand binding to cell membrane preparations containing recombinant human serotonin receptor (5-HT) subtypes. A 40% 2-propanol extract of black cohosh was tested against 10 subtypes of the serotonin receptor, revealing the presence of compounds with strong binding to the 5-HT$_{1A}$, 5-HT$_{1D}$, and 5-HT$_{7}$ subtypes. Subsequent binding studies were carried out using 5-HT$_{1A}$ and 5-HT$_{7}$ receptors because of their association with the hypothalamus, which has been implicated in the generation of hot flashes. The black cohosh 40% 2-propanol extract inhibited [$^{3}$H]lysergic acid diethylamide (LSD) binding to the human 5-HT$_{7}$ receptor (IC$_{50}$ = 2.4 ± 0.4 $\mu$g/mL) with greater potency than binding of [$^{3}$H]-8-hydroxy-2-(di-*N*-propylamino)tetralin to the rat 5-HT$_{1A}$ receptor (IC$_{50}$ = 13.9 ± 0.6 $\mu$g/mL). Analysis of ligand binding data indicated that components of a black cohosh methanol extract functioned as a mixed competitive ligand of the 5-HT$_{7}$ receptor. In addition, a black cohosh methanol extract elevated cAMP levels in 293T-5-HT$_{7}$-transfected HEK cells, suggesting the extract acted as a partial agonist at the receptor. The elevation in cAMP mediated by the black cohosh extract could be reversed in the presence of the antagonist methiothepin, indicating a receptor-mediated process. These data suggest that reductions in hot flashes in some women taking black cohosh may not be due to estrogenic properties. This study identifies other possible biological targets of black cohosh that could account for reported biological effects.

**KEYWORDS:** ***Actaea racemosa*** **L.;** ***Cimicifuga racemosa*** **(L.) Nutt.; black cohosh; serotonin receptor; hot flashes; estrogen receptor**

## INTRODUCTION

Steroidal hormone therapy has been routinely employed to relieve the symptoms associated with the decline in endogenous estrogens that are characteristic of menopause. Halting of the Prempro arm of the Women's Health Initiative due to increases in breast cancer, coronary heart disease, stroke, and pulmonary embolism necessitates investigations into alternative therapies for the alleviation of menopausal hot flashes (*1*). Extracts of the black cohosh rhizome (*Actaea racemosa* L., *Cimicifuga racemosa* (L.) Nutt.) have been used since the 1950s and tested

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successfully in several clinical trials for mitigating hot flashes (*2−9*). The mechanism of action for black cohosh has been presumed to involve hormonal signaling through the estrogen receptor (ER) or related targets because this was the pathway through which other hormone replacement therapies (HRT) acted to reverse menopausal symptoms. However, evidence supporting the estrogenic activity of black cohosh is inconsistent. Although early studies reported that black cohosh contained an estrogen receptor ligand that mitigated surges in luteinizing hormone (LH), a response that may have some association with the onset of a hot flash, more recent in vitro and in vivo studies have not shown estrogen-like effects (*9−18*). Formononetin, the ER ligand originally reported, was later determined to be an adulterant of black cohosh extracts (*19*, *20*). Moreover, ER binding studies performed with metabolized black cohosh extracts have failed to reveal any ligands of the ER (*18*). Furthermore, a recent animal study demonstrated that black cohosh does not initiate mammary tumor stimulating effects, endometrial proliferation, or hormone level changes in ovariectomized rats (*21*). These data support the theory that black cohosh might relieve menopausal hot flashes by a mechanism different from HRT; however, no such pathway has been confirmed.

In this study, the mechanism of action of black cohosh was investigated. First, ovariectomized rats were administered a 40% 2-propanol extract of black cohosh [4, 40, and 400 mg/(kg·day)] with or without estradiol [50 $\mu$g/(kg·day)] to determine if any estrogenic, antiestrogenic, or additive estrogenic effects could be detected. When no clear indications of estrogenic or antiestrogenic activity were discerned, other potential mechanisms were explored. Because black cohosh had previously been reported to reduce LH levels (*9*), mechanisms for potential LH reduction were considered as possible pathways through which black cohosh might reduce hot flashes. The theory that LH may be involved in the etiology of the hot flash was introduced in the 1970s (*22*, *23*), and although this gonadotropin is no longer considered to be the trigger for hot flashes, the connection has been documented (*24−26*). Serotonin receptors are known to at least partially control hot flashes and are present in the hypothalamus (*27−30*), the area of the brain modulated by estrogen in a negative inhibition pathway to reduce LH secretion from the pituitary. Serotonin selective reuptake inhibitors have also received attention for reducing hot flashes in menopausal women for whom HRT is contraindicated and in men who received androgen deprivation therapy (*31−34*). For these reasons, serotonin receptors were investigated as potential targets for black cohosh. This paper describes the binding of several hydroalcoholic extracts of black cohosh to several subtypes of the 5-HT receptor. Intracellular levels of cAMP were also measured to determine if black cohosh acted as an agonist or antagonist of the receptor. As a result, a new biological target of black cohosh that is independent of the estrogen receptor has been identified.

## MATERIALS AND METHODS

**Materials and Reagents.** All chemicals and reagents were purchased from Fisher (Hanover Park, IL) or Sigma (St. Louis, MO) unless otherwise indicated. All cell culture media were obtained from Life Technologies (Carlsbad, CA). FBS was acquired from Atlanta Biologicals (Norcross, GA). [$^3$H]Lysergic acid diethylamide (LSD), [$^3$H]-hydroxytryptamine (5-HT), and [$^3$H]-8-hydroxy-2-(di-*N*-propylamino)-tetralin (8-OH-DPAT) were obtained from NEN Life Science Products (Boston, MA). Plant materials were acquired as previously described (*35*). The air-dried, milled roots/rhizomes of *C. racemosa* were exhaustively extracted by percolation with 100% methanol, 75% ethanol, or 40% 2-propanol and then dried to powder form.

**Animals.** Guidelines established by our institutional Animal Care and Use Committee and state and federal regulations were followed for all procedures. The protocol complied with the Guide for the Care and Use of Laboratory Animals, and the facilities were approved by the Association for the Assessment and Accreditation of Laboratory Animal Care. Female ovariectomized rats weighing ∼200 g were received at 7 weeks of age from Harlan (Indianapolis, IN). Following a 3-day acclimation, the rats were weighed and housed in groups of three. Rat cages were arranged randomly to limit variation based on temperature and light. They were maintained in barrier rooms under a 12/12-h light/dark cycle, with a temperature of 22 °C and a relative humidity of 50%.

**Diets and Treatment.** All rats consumed a Harlan/Teklad Global 16% protein rodent diet (Indianapolis, IN), which contains no alfalfa or soybean meal. The diet must contain low levels of phytoestrogens to limit variation in results based on diet. Access to food was unrestricted, and water was administered using an automated watering system. The black cohosh 40% 2-propanol extract and the negative control, 1% carboxymethylcellulose (CMC), were administered by intestinal gavage. A 40% 2-propanol extract was chosen because similar preparations have been used in human clinical trials. 17$\beta$-Estradiol was suspended in sesame oil, and estradiol or oil alone was administered subcutaneously. The black cohosh extract was dissolved in a 1% CMC solution to yield concentrations of 2, 20, or 200 g/L. The 17$\beta$-estradiol was dissolved in the minimum amount of ethanol and then suspended in sesame oil to a concentration of 100 mg/L. The treatment groups were arranged as follows ($n = 6$): group 1, sesame oil and 1% CMCC; group 2, 50 $\mu$g/(kg·day) 17$\beta$-estradiol and 1% CMC; group 3, sesame oil and 4 mg/(kg·day) black cohosh; group 4, sesame oil and 40 mg/(kg·day) black cohosh; group 5, sesame oil and 400 mg/(kg·day) black cohosh; group 6, 50 $\mu$g/(kg·day) 17$\beta$-estradiol and 4 mg/(kg·day) black cohosh; group 7, 50 $\mu$g/(kg·day) 17$\beta$-estradiol and 40 mg/(kg·day) black cohosh; group 8, 50 $\mu$g/(kg·day) 17$\beta$-estradiol and 400 mg/(kg·day) black cohosh.

**Determination of Uterine Weight.** Twenty-four hours after the final treatment, rats were killed by $CO_2$ asphyxiation. At necropsy, uteri were collected, trimmed of fat and connective tissue, cut open, drained of intrauterine fluid, weighed, frozen in 1.5-mL cryogenic vials on dry ice, and stored at −70 °C.

**Vaginal Cellular Differentiation Analysis.** Vaginal cytology smears were taken and scored daily to monitor cellular differentiation. A smear was performed on all rats before dosing to establish a baseline and to confirm that all ovariectomized smears showed no cornification. Vaginal smears were taken daily using an eyedropper containing 8.5 g/L NaCl, placed on ringed slides, and observed under a light microscope using a 10× eyepiece and a 10× objective. The same technician, unblinded, read smears immediately, and they were identified as leukocytes, nucleated, or cornified epithelial cells. A raw score on a scale of 1−5 was assigned for cell populations ranging from entirely leukocytes (indicating a proestrous stage) to entirely cornified (indicating a diestrous stage).

**Cell Culture Conditions.** The human 5-HT$_7$-transfected Chinese hamster ovary (CHO) cell line was generously provided by David Sibley (National Institutes of Health, Bethesda, MD) and cultured with Ham's F-12 medium, containing FBS (10%), 1 mM MEM sodium pyruvate, 50 mg/mL gentamycin, and 50 units/mL of penicillin/streptomycin. The rat 5-HT$_{1A}$-transfected LZD$_7$ (transfectant of the Ltk mouse fibroblast) cell line was provided by Dr. Paul R. Albert (McGill University, Montreal, PQ, Canada). LZD$_7$ cells were cultured with MEM with nonessential amino acids containing FBS (10%) and antibiotic−antimytotic (1%). The 5-HT$_7$-transfected HEK293 (human epithelial kidney) cell line was provided by Dr. Mark Hamblin (University of Washington, Seattle, WA). The HEK293 5-HT$_7$-transfected cells were cultured in DMEM supplemented with puromycin (10 $\mu$g/mL), antibiotic−antimycotic (1%), and FBS (10%). Four days before cAMP assays were performed, the culture medium was replaced with Cellgro Complete serum-free medium from Mediatech (Herndon, VA).

**Membrane Preparation.** Cells (human 5-HT$_7$ CHO or rat 5-HT$_{1a}$ Ltk mouse fibroblast) were plated into dishes (150 mm × 10 mm) and cultured to confluence in order to collect membranes as previously described (*36*). A hypotonic buffer (15 mM Tris, 1.25 mM $MgCl_2$,

and 1 mM EDTA, pH 7.4) was added to the dishes and incubated at 4 °C for 15 min, the cells were scraped from the dishes, and the lysate was centrifuged. The hypotonic buffer was removed and the membrane pellet resuspended in TEM buffer (75 mM Tris, 1 mM EDTA, 12.5 mM $MgCl_2$, pH 7.4). The cell membranes were homogenized and centrifuged twice at 12000*g* for 20 min. The pellets were dissolved in TEM buffer and were stored at −70 °C. Protein concentrations were determined according to the Lowry method using bovine serum albumin as the standard.

**Serotonin Receptor Binding Assays.** Initial radioligand binding studies were performed by Panlabs (Bothell, WA) as previously described for serotonin receptor subtypes 1A, 1B, 1D, 2A, 2B, 2C, 3, 5A, 6, and 7 (*37*−*42*). For the 5-$HT_7$ receptor, additional assays were performed with minor modifications using human recombinant CHO cell membrane and [$^3$H]LSD (5 nM) in an incubation buffer (75 mM Tris-HCl, 1.25 mM $MgCl_2$, 1 mM EDTA, pH 7.4) (*43*). Assays with the rat 5-$HT_{1A}$ were performed with recombinant $LZD_7$ cell membranes using [$^3$H]-8-OH-DPAT (5 nM) incubated at 37 °C for 60 min in incubation buffer (75 mM Tris-HCl, 1.25 mM $MgCl_2$, 1 mM EDTA, 0.1% L-ascorbic acid, 10 μM pargyline, pH 7.4) (*36*, *44*, *45*). After a 1-h incubation at 37 °C, the mixtures for both receptors were filtered over 934-AH Whatman filters that had been presoaked in 0.5% polyethylenimine (PEI) and washed two times in ice-cold 50 mM Tris buffer (pH 7.4) using a 96-well Tomtec-Harvester (Orange, CT). Each filter was dried, suspended in Wallac microbeta plate scintillation fluid (PerkinElmer Life Sciences, Boston, MA), and counted with a Wallac 1450 Microbeta liquid scintillation counter (PerkinElmer Life Sciences, Boston, MA). 5-Hydroxytryptamine (serotonin, 5-HT) (250 nM) was used to define nonspecific binding, which accounted for <10% of total binding. The percent inhibition of [$^3$H]ligand bound to each 5-HT receptor was determined as $[1 - (dpm_{sample} - dpm_{blank})/(dpm_{DMSO} - dpm_{blank})] \times 100$. The data represent the average ± SD of at least triplicate determinations.

**Receptor Kinetics.** The mechanism of receptor binding was characterized using the methods described above with the modification that concentrations of [$^3$H]LSD ranged from 0.5−6 nM and the black cohosh methanol extract ranged from 0−20 μg/mL. The $K_d$ and $K_i$ values were determined using the equations for a single-substrate single-inhibitor model and the software available in the Sigmaplot Enzyme Kinetics module (SPSS, Chicago, IL). Direct binding and reciprocal plots were analyzed to determine the receptor−ligand interaction. Full and partial competitive, uncompetitive, and noncompetitive inhibition models were all evaluated and ranked according to the best-fit based on their $R^2$ and AIC values.

**Intracellular cAMP Assays.** Elevation of intracellular cAMP was assayed as previously described with minor modifications (*36*). HEK293 human transfected 5-$HT_7$ cells were grown for 4 days in serum-free media and then plated in poly(D-lysine)-coated 12-well plates (100 × $10^4$ cells/well) and left overnight. The following day, the cells were washed twice with 2 mL of incubation buffer (150 nM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, 500 μM isobutylmethylxanthine, 1 μM ascorbic acid, 10 μM pargyline, pH 7.4) and then incubated for 20 min at 37 °C. The compounds and extracts were added at concentrations 1000-fold higher than their final concentrations in DMSO and incubated at 37 °C for 10 min. The reactions were terminated by aspirating the buffer and adding 1 mL of boiling water to lyse the cells. The cells were then subjected to four rapid freeze−thaw cycles using a methanol and dry ice bath and a 37 °C water bath. An aliquot of cell lysate (50 μL) was then transferred into the cAMP binding assay using the Amersham kit (TRK 432). In the separation step, Whatman filters were used instead of charcoal as described in the binding assay protocol (*36*).

## RESULTS

**Body Weight.** Ovariectomized animals treated with estradiol maintained a similar body weight throughout the 2 week study (**Table 1**). However, animals that received the vehicle control (DMSO) showed increases in body weight. Therefore, the maintenance of body weight was interpreted as an indication of estrogenic activity. Animals receiving DMSO or black cohosh alone [4, 40, and 400 mg/(kg•day)] showed increases in body weight of ∼20 mg over the course of the study. This indicated that black cohosh did not act like estrogen in that it failed to prevent body weight gain. Animals treated with either estradiol or black cohosh plus estradiol experienced no significant weight change. Therefore, black cohosh did not act as an antiestrogen to reverse estradiol-mediated maintenance of body weight.

**Table 1.** Uterine and Body Weights of Ovariectomized Rats Treated with Black Cohosh 40% 2-Propanol Extract, Estradiol, or Solvent Control[a]

| treatment group and dose | uterine wt (g) | body wt[b] (g) |
| --- | --- | --- |
| vehicle | 0.11 ± 0.04 | 224 ± 7 |
| estradiol [50 μg/(kg•day)] | 0.36 ± 0.03 | 206 ± 9 |
| black cohosh [4 mg/(kg•day)] | 0.10 ± 0.01 | 216 ± 9 |
| black cohosh [40 mg/(kg•day)] | 0.11 ± 0.02 | 219 ± 7 |
| black cohosh [400 mg/(kg•day)] | 0.11 ± 0.02 | 224 ± 9 |
| black cohosh [4 mg/(kg•day)] + estradiol [50 μg/(kg•day)] | 0.35 ± 0.06 | 199 ± 10 |
| black cohosh [40 mg/(kg•day)] + estradiol [50 μg/(kg•day)] | 0.36 ± 0.02 | 209 ± 7 |
| black cohosh [400 mg/(kg•day)] + estradiol [50 μg/(kg•daay)] | 0.33 ± 0.02 | 206 ± 8 |

[a] Values are the means ± SD of six animals. Animals were treated with black cohosh every day for 14 days. Experimental details are given under Materials and Methods. [b] Average weight of rats at the end of 2 weeks.

**Uterine Weight.** Uteri removed from the rats treated with black cohosh at all doses tested [4, 40, and 400 mg/(kg•day)] showed no increase in weight compared to DMSO controls. The uteri taken from animals that received black cohosh in conjunction with estradiol had similar uterine weights compared with estradiol treatment alone, and this weight gain was not attenuated by the presence of the black cohosh 2-propanol extract (**Table 1**). These data demonstrate that black cohosh extract does not provide estrogenic stimulation to the uterus and does not mitigate the uterine proliferative effects of estradiol.

**Vaginal Cellular Differentiation.** The inner lining of the vaginal tract is responsive to estrogen such that its presence causes the epithelial cells to differentiate from leukocytes into cornified cells. Estradiol [50 μg/(kg•day)] caused maturation of cells to their fully cornified state within 3 days; however, black cohosh did not stimulate cells beyond the leukocyte stage. Once the vaginal cells of the estradiol-treated rats reached their fully differentiated state, the population remained completely cornified, resulting in no error in the *y* value. Black cohosh also did not block differentiation in animals treated with both the extract and estradiol (data not shown). **Figure 1** shows only the maturation of cells from the highest dose of black cohosh [400 mg/(kg•day)], which did not differ significantly from the DMSO control.

**Binding of Black Cohosh Extract to Different Subtypes of the Serotonin Receptor.** Because black cohosh extracts did not demonstrate any discernible estrogenic behavior in any of the in vitro assays (*15*) or in the ovariectomized rat model, other biological targets were studied. Serotonin receptors were investigated on the basis of their neuroendocrine links to menopausal hot flashes, and a previous literature report that *Cimicifuga foetida* bound to the 5-$HT_{1A}$ receptor (*46*). Serotonin receptor binding was evaluated to determine if black cohosh displayed other pharmacological properties.

A 40% 2-propanol extract of black cohosh was screened by Panlabs (Bothell, WA) to determine if the extract contained any potential ligand(s) of the serotonin receptor subtypes 1A, 1B, 1D, 2A, 2B, 2C, 3, 5A, 6, and 7. The extract (250 μg/mL)

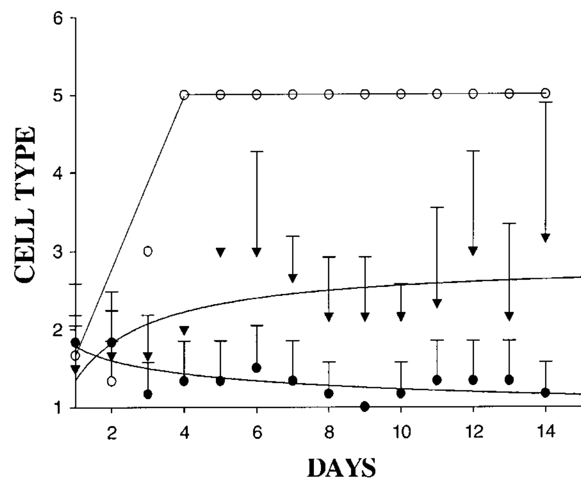


**Figure 1.** Vaginal cellular differentiation in response to vehicle control, estradiol, and black cohosh [400 mg/(kg·day)]: (●) vehicle; (○) estradiol; (▼) black cohosh [400 mg/(kg·day)]. Vaginal smears were acquired by aspiration daily for 14 days. Numbers on the *y*-axis represent the numerical scale assigned according to the predominate cell type, with 1 being completely undifferentiated leukocytes, 3 being mostly nucleocytes, and 5 being complete estrogenic cornification. Values are the means ± SD, $n = 6$.

produced the greatest inhibition of receptor binding for the 1A, 1D, and 7 receptors (>95%). A black cohosh 2-propanol extract bound (>50%) to all subtypes except for 5-HT$_{2A}$ and 5-HT$_3$ (data not shown). The black cohosh 2-propanol extract did not significantly alter radioligand binding to the serotonin transporter (data not shown).

Because 5-HT$_{1A}$ and 5-HT$_7$ are both located in the hypothalamus,where they might be involved in hormonal regulation, and because they both have similar drug sensitivities, dose–response studies were conducted to further distinguish the relative binding potency of black cohosh extract for only these two receptors. To validate the method, serotonin (5HT) was tested as an inhibitor of [$^3$H]LSD binding for the 5-HT$_7$ receptor and [$^3$H]-8-OH-DPAT binding for the 5-HT$_{1A}$ receptor. Experiments generated $K_i$ values of 1.5 nM for 5-HT$_7$ and 2.8 nM for 5-HT$_{1A}$ that were consistent with the literature (*36*, *47*). Three hydroalcoholic extracts were compared to determine which extraction procedure resulted in the greatest percent inhibition of binding of a radiolabeled compound to the serotonin receptors: a 100% methanol extract, a 40% 2-propanol extract, or a 75% ethanol extract. Parts A and B of **Figure 2** show the percent inhibition of binding for the three extracts studied for the 5-HT$_7$ and 5-HT$_{1A}$ receptors, respectively. The black cohosh extracts produced a greater inhibition of radiolabeled ligand binding to the 5-HT$_7$ receptor relative to the 5-HT$_{1A}$ receptor. The methanol extract displaced radioligands from the 5-HT$_{1A}$ (IC$_{50}$ = 2.5 ± 0.6 $\mu$g/mL) and 5-HT$_7$ (2.2 ± 0.2 $\mu$g/mL) receptors equally well. However, the 75% ethanol and the 40% 2-propanol extracts inhibited binding of 8-OH-DPAT to the 5-HT$_{1A}$ (IC$_{50}$ = 13 ± 2; 14 ± 1 $\mu$g/mL) receptor less effectively than binding of LSD to the 5-HT$_7$ receptor (IC$_{50}$ = 3.1 ± 0.5; 2.4 ± 0.4 $\mu$g/mL). Because the black cohosh methanol extract exhibited the highest binding potency for both receptors studied, only this extract was used for further analyses.

**Receptor Binding Mechanism.** To determine if a black cohosh methanol extract functioned as a competitive, noncompetitive, uncompetitive, or mixed inhibitor(s) of LSD binding to the 5-HT$_7$ receptor, LSD binding experiments were performed in the absence and presence of 10 and 20 $\mu$g/mL of the extract. **Figure 3A** represents a direct binding isotherm plot of triplicate data for each concentration of black cohosh and [$^3$H]LSD. Saturable binding with increasing concentrations of [$^3$H]LSD cannot be achieved in the presence of black cohosh methanol extract, indicating that the ligand(s) contained in the extract do not act as a full competitor(s) of LSD for the receptor binding site. When reciprocal plots (**Figure 3B**) were analyzed, the data best fit a model for mixed inhibition in which the ligand(s) present in black cohosh could bind to the receptor binding site alone but could also bind in the presence of the known ligand, [$^3$H]LSD. The $K_d$ of LSD in this study was found to be 0.9 ± 0.3 nM. The model for how the unknown ligand(s) in black cohosh methanol extract bind(s) to the receptor in the presence and absence of LSD is shown schematically in **Figure 4**.

**Cyclic Adenosine Monophosphate (cAMP) Elevation Studies.** 5-HT$_7$ receptors are coupled to stimulatory G-proteins. Upon agonist occupancy of the receptor, the G$_\alpha$ subunit of the G-protein dissociates and activates adenylate cyclase to catalyze the formation of cAMP. The HEK293 stably transfected 5-HT$_7$ cells produced cAMP in the presence of serotonin (**Figure 5**). This stimulation could be eradicated by co-incubation of serotonin with the receptor antagonist methiothepin. HEK/5-HT$_7$ cells were studied to determine if compounds in the black cohosh methanol extract acted as agonists or antagonists of the receptor. Incubations of the black cohosh methanol extract with HEK/5-HT$_7$ cells demonstrated that the extract contained serotonin receptor agonist(s) that increased intracellular levels of cAMP. Black cohosh extract was also co-incubated with 5-HT to determine if any antagonist(s) was (were) found within the plant material, but no attenuation of the 5-HT-stimulated production of cAMP was observed at doses of 40 $\mu$g/mL (data not shown). Finally, the black cohosh methanol extract was incubated in the presence of the antagonist, methiothepin, and the formation of cAMP was reduced, indicating this process is receptor mediated.

## DISCUSSION

Concerns regarding the risks of hormone replacement therapy have increased the interest in alternatives (*48*). Although HRT was once thought to reduce the risks of cardiovascular disease, osteoporosis, and stroke, the Women's Health Initiative found a net increase in adverse effects in women taking HRT for 5.2 years (*1*). Botanical dietary supplements are under investigation as alternatives to HRT, and understanding their mechanism(s) of action will facilitate their safe and effective use (*49*, *50*).

Five randomized placebo-controlled clinical trials have been completed using black cohosh extracts, and four of them showed improvement in vasomotor symptoms according to the Kupperman Index (*4*, *8*, *51*, *52*). Although women in one trial did not experience any reduction in hot flashes (*53*), this might have been caused by the simultaneous administration of tamoxifen. A similar clinical trial was performed with coadministration of black cohosh and tamoxifen for 12 months and found a significant reduction in hot flashes, suggesting that prolonged use of black cohosh is necessary to mitigate tamoxifen-induced hot flashes (*52*).

Conflicting reports exist in the literature regarding the presence of estrogenic compounds in black cohosh. Early in vitro data indicated estrogenic effects such as induction of MCF-7 cellular proliferation and displacement of estradiol from specific antibodies that recognize estrogen moieties (*14*, *16*). However, one study reported that the active compound responsible for MCF-7 proliferation, fukinolic acid, was not potent

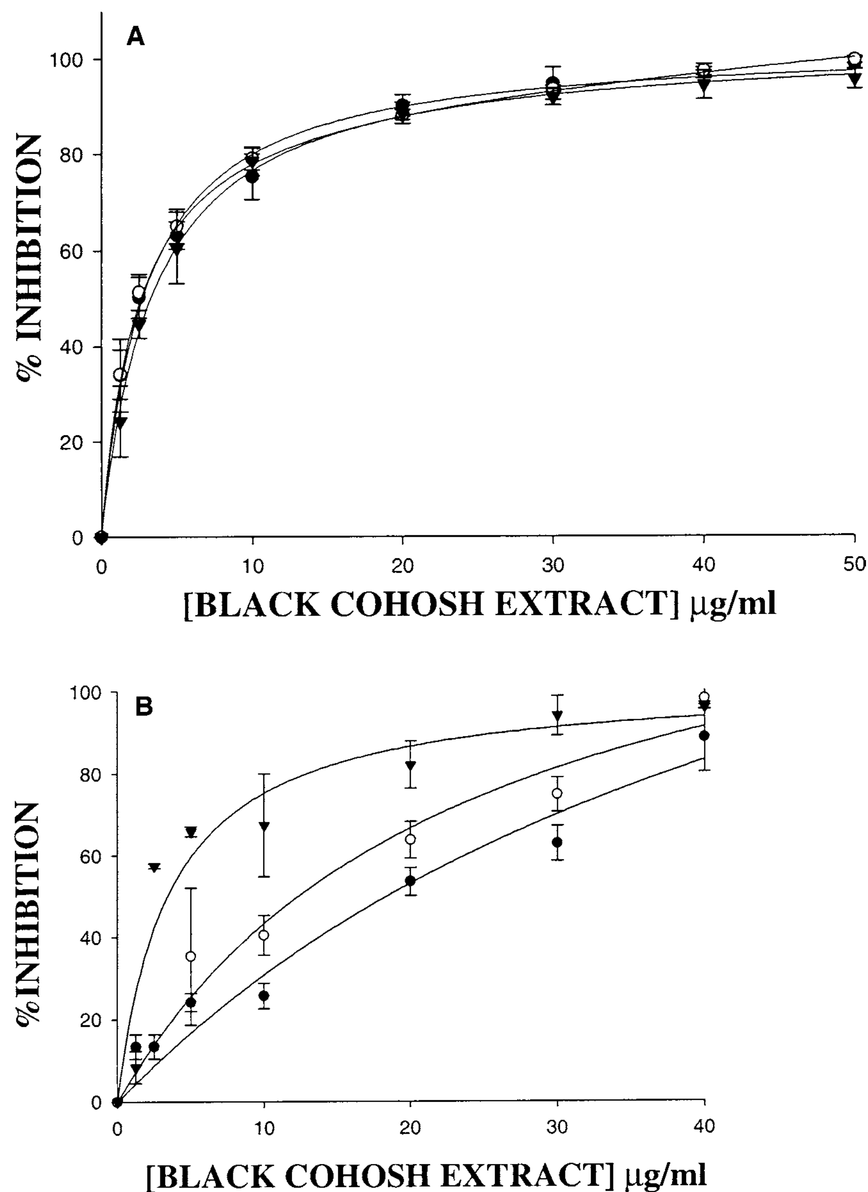


**Figure 2.** Serotonin receptor binding of black cohosh to (A) human 5-$HT_7$ transfected CHO membranes in the presence of [$^3$H]LSD and (B) rat 5-$HT_{1A}$ transfected Ltk membranes in the presence of [$^3$H]-8-OH-DPAT: (A) the three extract preparations tested all bound with equal ability [(○) 40% 2-propanol extract, (●) 75% ethanol extract, and (▼) 100% methanol extract]; (B) the methanol extract bound with the highest affinity followed by the 2-propanolic extract and then the ethanolic extract [(○) 40% 2-propanol extract, (●) 75% ethanol extract, and (▼) 100% methanol extract].

enough to explain the therapeutic efficacy of black cohosh (*14*). It was reported with animal studies that vaginal cellular cornification increased, but the uteri did not increase in weight in the black cohosh-treated group (*16*). Another group reported that black cohosh had estrogenic properties because it reduced LH, but the putative active compound showed no biological activity when administered alone (*11*, *12*). Formononetin, an estrogen receptor ligand reportedly found in black cohosh, was later proven to be an adulterant of the plant material (*13*, *19*, *20*). Subsequent investigations of black cohosh for estrogenic properties have identified no ligands and no estrogen-inducible gene up-regulation in cultured mammalian cell lines (*15*, *18*, *54*). Hydrolysis of alcoholic preparations of black cohosh did increase displacement of radiolabeled estrogen from the estrogen receptors alpha and beta, and this process may mimic metabolism in the body by gut flora. However, evidence to support that black cohosh produced in vivo estrogenic stimulation is not supported by this study or previous studies (*55*, *56*). However, some authors suspect that an unidentified ligand of an unidentified estrogen receptor gamma may be present in black cohosh extracts (*55*). One paper reported antiestrogenic properties of black cohosh that apparently were not estrogen receptor mediated and inconsistent with other literature where black cohosh was not able to prevent estradiol-induced expression of alkaline phosphatase activity in Ishikawa cells (*15*, *57*).

Three recent animal studies conducted using black cohosh extracts found no estrogenic increases in uterine weight, stimulation of vaginal cornification, or proliferation of the mammary gland or increases in prolactin, follicle-stimulating hormone, or luteinizing hormone (*10*, *21*, *56*). One of these animal studies was conducted for only 3 days, even though most women taking black cohosh do not report a reduction in symptoms for at least 2 weeks (*10*). The second study was designed to determine whether the extract induced tumors, and lower doses of black cohosh were used relative to the current study [0.714, 7.14, or 71.4 mg/(kg·day)] (*21*). The third study was conducted for 3 months with 33 mg/day of a black cohosh aqueous/ethanol extract called BNO 1055 (*56*). This study found

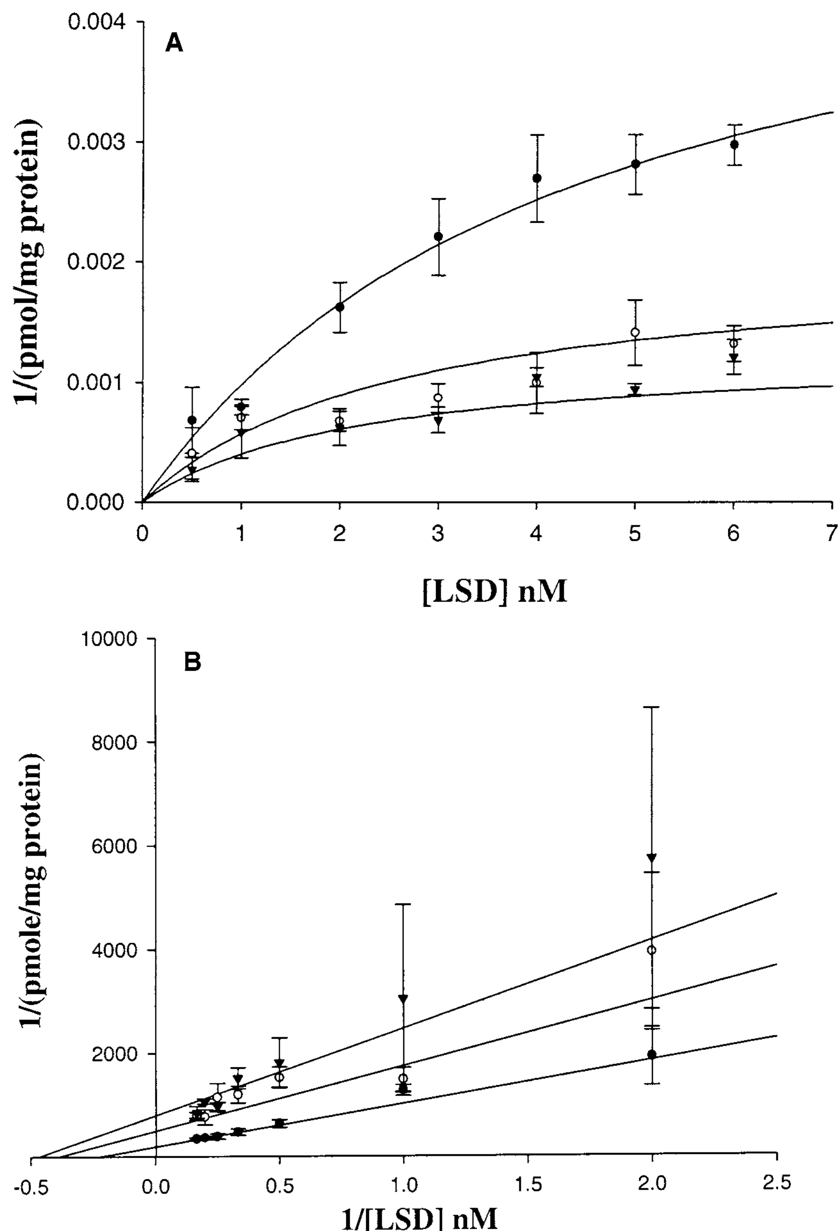


**Figure 3.** Binding plot displaying the effect of black cohosh on the binding of [$^3$H]LSD to the 5-HT$_7$ receptor. (A) Direct binding plots are shown for increasing concentrations of [$^3$H]LSD incubated with increasing concentrations of black cohosh. [$^3$H]LSD was not able to bind to all of the receptor binding sites in the presence of black cohosh, indicating a mixed full inhibition: (●) no black cohosh; (○) 10 $\mu$g/mL black cohosh; (▼) 20 $\mu$g/mL black cohosh. (B) Reciprocal plots of the data in (A) illustrate that black cohosh methanol extract acts as a mixed competitor of the 5-HT$_7$ receptor in the presence of [$^3$H]LSD. The plot reveals that a black cohosh methanol extract contains a mixed ligand of the serotonin receptor such that both ligands can be simultaneously bound. The $K_d$ for [$^3$H]LSD was 0.9 nM, the $K_i$ of black cohosh was 6.1 $\mu$g/mL, and the $\alpha$ factor was 2.9: (●) no black cohosh; (○) 10 $\mu$g/mL black cohosh; (▼) 20 $\mu$g/mL black cohosh.

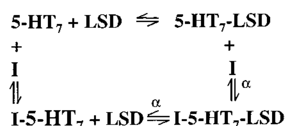


**Figure 4.** Scheme depicting the mixed competitive inhibition model for the interaction of black cohosh methanol extract and the inhibitor (I) with the serotonin receptor 5-HT$_7$ in the presence of [$^3$H]LSD.

no increase in uterine weight and no increase in IGF-1 or ER$\beta$ gene expression in the uterus (*56*). The study did see a reduction in abdominal fat deposition, an indication of estrogenic stimulation, but no reduction in total body weight gain, consistent with the current study. One interesting finding was that black cohosh improved bone mineral density of the tibia, suggesting that the extract may contain compounds useful for preventing osteoporosis (*56*). We currently demonstrate that black cohosh was not estrogenic when animals were treated for 2 weeks with higher doses [4, 40, and 400 mg/(kg·day)] than used previously. In addition, we evaluated and found no antiestrogenic properties by coadministering estradiol with black cohosh in ovariectomized rats [50 $\mu$g/(kg·day) estradiol + 4, 40, and 400 mg/(kg·day) black cohosh 40% 2-propanol extract]. No additive estrogenic induction was observed when black cohosh and estradiol were administered simultaneously. Therefore, our results contradict previously reported in vitro data that identified antiestrogenic inhibition of MCF-7 cellular proliferation and inhibition of an estrogen-inducible yeast gene from treatment with black cohosh extract (*57*).

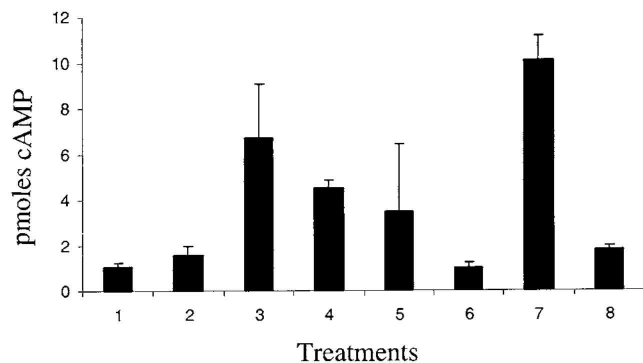


**Figure 5.** Cyclic AMP intracellular levels of human 5-$HT_7$-transfected HEK293 cells after treatment with black cohosh: (1) untreated cells; (2) DMSO; (3) forskolin (20 $\mu$M); (4) 5-HT (10 $\mu$M); (5) 5-carboxytryptamine (10 $\mu$M); (6) 5-HT (10 $\mu$M) + methiothepin (5 $\mu$M); (7) black cohosh (40 $\mu$g/mL); (8) black cohosh (40 $\mu$g/mL) + methiothepin (5 $\mu$M). 5-HT, 5-CT, and forskolin stimulated cAMP production. 5-HT-stimulated cAMP could be reversed in the presence of methiothepin. Receptor-mediated cAMP formation from black cohosh extract could also be reversed with methiothepin.

Because all of our data indicated that black cohosh functioned in an estrogen-independent manner, another biological target was selected on the basis of the following rationale. Although HRT alleviates hot flashes, a decline in estrogen itself is not the trigger of hot flashes because levels do not differ significantly between symptomatic and asymptomatic women. Also, levels of estrogen remain low for the remainder of a woman's life, but hot flashes eventually cease (*48*). Two studies conducted with black cohosh suggest that the extract might act through the serotonin pathway (*45*, *58*). One study reported that a water extract of *C. foetida* L. bound to the serotonin receptor 5-$HT_{1A}$ (*46*); the other study found that a methanol extract of *Cimicifuga* rhizomes inhibited 5-HT-induced diarrhea in mice (*58*). In addition to this evidence, there are a number of documented connections between the estrogenic and serotonergic systems. First, when estrogen levels fall, the amount of tryptophan hydroxylase in the body responsible for converting tryptophan into serotonin is also lowered, limiting the formation of serotonin (*59*, *60*). Second, the level of serotonin in the bloodstream of menopausal women declines when estrogen declines (*61*). HRT is capable of augmenting this diminished serotonergic activity (*62*−*64*). Clinical trials using selective serotonin re-uptake inhibitors (SSRIs) have shown success in reducing menopausal hot flashes in both healthy women and cancer patients taking selective estrogen receptor modulators (*31*, *32*, *34*).

Results from our laboratory and others indicated that black cohosh extracts contained serotonin receptor ligands. The black cohosh ligand(s) had the highest affinity for the 5-$HT_{1A}$, 5-$HT_{1D}$, and 5-$HT_7$ receptors at a high dose (250 $\mu$g/mL). Little is known about the 5-$HT_{1D}$ receptor (*65*), but it has been suggested that it has pharmacological properties similar to those of the 5-$HT_{1B}$ receptor. The physiological functions of the 5-$HT_{1B}$ receptor are poorly understood because there are no selective compounds for this receptor (*65*). The 5-$HT_{1A}$ and 5-$HT_7$ receptors were further examined because they are both found in the hypothalamus, a key area for thermoregulation. Indeed, the administration of a 5-$HT_{1A}$ agonist into the anterior hypothalamus of a rat induces hypothermia (*66*). Our results showed that the extract was somewhat selective for the 5-$HT_7$ receptor because all three extracts tested (100% methanol, 40% 2-propanol, and 75% ethanol) bound to the receptor with $IC_{50}$ values of $\leq$3.12 $\mu$g/mL. Serotonin has been shown to inhibit the secretion of LH from the hypothalamus through the 5-$HT_{1A}$ (*29*) receptor, and black cohosh has previously been reported to reduce LH concentrations (*9*). Serotonin is known to control the pulsatile release of LH by acting on the hypothalamus and, using an ovariectomized rat model, serotonin receptors have been found that terminate directly onto luteinizing hormone releasing hormone (LhRh) neurons and inhibit the secretion of LH from the pituitary (*27*−*30*). This is of interest because the selective agonist, 8-OH-DPAT, used to determine that 5-$HT_{1A}$ receptors were involved in LH reduction, is also a partial agonist of the 5-$HT_7$ receptor, the most recently identified serotonin receptor, and these data at least partially implicate both receptors as being involved in the regulation of LH release by controlling the hypothalamus (*67*). Also, selective 5-$HT_7$ receptor ligands have not been identified, and black cohosh may contain lead compound(s) that could be used to develop more selective ligands for the receptor.

The black cohosh methanol extract was compared to a 40% 2-propanol extract and a 75% ethanol extract, and the methanol extract showed the greatest inhibition of binding for the 5-$HT_7$. Therefore, further experiments were performed with only the methanol extract and the 5-$HT_7$ receptor. Black cohosh extract was shown to interact with the 5-$HT_7$ receptor by a mixed competitive model. The extract was capable of binding to the receptor in the presence and absence of [$^3$H]LSD.

Determination of cAMP levels showed that a black cohosh methanol extract acted as a 5-$HT_7$ agonist and stimulated the G-protein coupled activation of adenylate cylase. This process is receptor mediated as demonstrated by the co-incubation of a black cohosh methanol extract with the antagonist methiothepin. These data are consistent with the idea that a treatment capable of increasing serotonergic activity in the body may help to reduce hot flashes, as shown by the clinical trials performed with menopausal women using SSRIs (*31*, *32*, *34*, *68*). Because the ligand(s) in black cohosh was (were) able to increase the amount of cAMP inside the cell, this supports the idea that the ligand, despite its mixed interaction with the receptor, has the same downstream effects as other competitive ligands.

SSRIs are currently used for the treatment of depression. An animal study using black cohosh extract used the tail suspension test in rats as a marker for antidepressant activity and found that black cohosh extract can produce behavior indicative of antidepressant activity (*69*). The positive control, imipramine, used in the animal study was a mixed inhibitor of the serotonin and norepinephrine transporters (*69*, *70*). These data are consistent with the theory that black cohosh may be stimulating serotonin receptors. In addition, these data demonstrate that black cohosh extract is capable of producing in vivo behavioral changes consistent with those seen by other serotonergic compounds.

These data indicate that black cohosh acts on serotonin receptors but not on estrogen receptors. Despite the lack of estrogenic effects, many clinical trials have found that black cohosh effectively reduces menopausal hot flashes, indicating that another target is possibly involved in the alleviation of hot flashes (*8*). Nonhormonal treatments may reduce menopausal symptoms without increasing a women's risk for developing breast cancer. Although the results presented do not show how black cohosh extracts alleviate hot flashes, another biological target has been identified for black cohosh, providing a possible alternative mechanism of action. Active compounds in the extract must be identified and characterized for their receptor binding and agonistic properties. Future studies will be conducted to determine if black cohosh actually reaches serotonin

receptors in the brain and has effects on hot flashes in women. These data demonstrate that black cohosh does not contain compounds that act as estrogens or antiestrogens in the ovariectomized rat model, but instead a partial agonist of the serotonin receptor is contained in the extract.

## ABBREVIATIONS USED

CR, *Actaea racemosa* L., *Cimicifuga racemosa* (L.) Nutt. (black cohosh); CHO, Chinese hamster ovary; CMC, carboxymethylcellulose; 5-CT, 5-carboxytryptamine (5-CT); DMSO, dimethyl sulfoxide; DPAT, 8-hydroxy-(2-(*N*,*N*-di[2,3-$^3$H])propylamino)tetralin; ER, estrogen receptor; FBS, fetal bovine serum; 5-HT, 5-hydroxytryptamine (serotonin); LH, luteinizing hormone; LSD, lysergic acid diethylamide.

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## NOTE ADDED AFTER ASAP POSTING

From the original posting of July 29, 2003, in the first paragraph "consistent" has been changed to "inconsistent". In the second paragraph following Figure 5, the phrase "are not poorly understood," has been changed to "are poorly understood". The revised version was posted July 31, 2003.

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