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Failure of Cannabinoid Compounds to Stimulate Estrogen Receptors

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ABSTRACT. Δ^9-Tetrahydrocannabinol (THC), the primary active compound in *Cannabis sativa* (marihuana), and other cannabinoid receptor agonists exert potent effects on luteinizing hormone and prolactin release in animal models and humans. Compounds possessing the tricyclic cannabinoid structure, including Δ^9-THC and cannabidiol, have been reported to interact with rodent uterine estrogen receptors in ligand binding assays. The present study tested the hypothesis that cannabinoid compounds produce a direct activation of estrogen receptors. We investigated whether cannabinoid compounds exhibit estrogen-induced mitogenesis in MCF-7 breast cancer cells. Under conditions in which 10 pM estradiol promoted MCF-7 cell proliferation, no response was observed with biologically relevant concentrations ($\leq$10 μM) of Δ^9-THC or its tricyclic analog desacetyllevonantradol. No response was observed with cannabidiol, a bicyclic cannabinoid compound that exhibits no cannabimimetic behavioral effects but has been reported to bind to the estrogen receptor *in vitro*. Δ^9-THC also failed to antagonize the response to estradiol under conditions in which the antiestrogen LY156758 (keoxifene; raloxifene) was effective. The phytoestrogen formononetin behaved as an estrogen at high concentrations, and this response was antagonized by LY156758. We also investigated the ability of cannabinoid compounds to stimulate transcription of an EREtkCAT reporter gene transiently transfected into MCF-7 cells. Neither Δ^9-THC, desacetyllevonantradol, nor cannabidiol stimulated transcriptional activity. We conclude that psychoactive or inactive compounds of the cannabinoid structural class fail to behave as agonists in appropriate assays of estrogen receptor responses *in vitro*. *Copyright © 1996 Elsevier Science Inc.*, BIOCHEM PHARMACOL 53;1:35–41, 1997.

KEY WORDS. breast cancer cells; cannabinoid analgesics; cannabinoid receptors; environmental estrogens; gene regulatory response elements; phytoestrogens; steroid hormone receptors; tetrahydrocannabinols

Δ^9-THC,§ the primary active compound in *Cannabis sativa* (marihuana), and other cannabinoid receptor agonists exert potent effects on luteinizing hormone and prolactin release in animal models and humans. The abundant literature on this subject has been reviewed recently [1–3]. It is currently recognized that the majority of the actions of cannabimimetic compounds on reproductive physiology may be the result of interactions of these drugs with CB_1 cannabinoid receptors present on neurons in the brain that ultimately control pituitary hormone release. Of interest, CB_1 cannabinoid receptor mRNA has also been found in the testes, ovaries, and uterus [4–6]. The pharmacology of CB_1 cannabinoid receptors in the brain has been reviewed recently [7–9]. To summarize, CB_1 cannabinoid receptors are G-protein-coupled receptors that utilize G_i and G_o in signal transduction pathways that inhibit adenylate cyclase and Ca^{2+} currents, and stimulate K^+ currents. Δ^9-THC exhibits nanomolar affinity for the CB_1 receptor, and elicits cannabimimetic behavioral responses in rodents, such as analgesia, hypothermia, catalepsy, and hypoactivity in an open field test. However, CBD, also a member of the cannabinoid structural class of compounds isolated from C. *sativa*, interacts with the CB_1 receptor with >100-fold lower potency than Δ^9-THC, and fails to elicit cannabimimetic behavioral responses in rodents. A synthetic cannabinoid agonist, DALN, binds to the CB_1 receptor with a 10-fold greater affinity than does Δ^9-THC, and exhibits similarly increased potency in the behavioral tests *in vivo*.

The interaction of cannabinoid compounds with estrogen receptors has been studied with conflicting results. Rawitch and colleagues [10], performing heterologous competition assays for [^{3}H]estradiol binding to rat uterine estrogen receptors in cytosol, found that 5 to 50 μM Δ^9-THC or 11-OH-Δ^9-THC could displace specific binding with a ceiling of about 22%. In that same study, the [^{3}H]estradiol-re-

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§ *Abbreviations:* CAT, chloramphenicol acetyl transferase; CBD, cannabidiol; DALN, desacetyllevonantradol; ERE, estrogen responsive element; HBSS, Hanks' balanced salt solution buffered with 25 mM HEPES; MEM, minimum essential medium; THC, tetrahydrocannabinol.

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ceptor complex cosedimented on a sucrose density gradient with [^{14}C]Δ^9-THC labeled peaks at 4S and 10.4S values. In contrast, Okey and Bondy [11] failed to observe any displacement of [^{3}H]estradiol-17β from 4S or 8S peaks of rodent uterine estrogen receptors by either 10 μM Δ^9-THC or *Cannabis* resin at an amount that would contribute 10 μM Δ^9-THC plus other cannabinoid compounds. As a control, 100 nM diethylstilbestrol completely displaced radioligand binding in those experiments. Chakravarty and Naik [12] were unable to find supportive evidence for a Δ^9-THC interaction with rat estrogen receptors in numerous tissues using *in vitro*, *in vivo* and *ex vivo* techniques. To further address this issue, Sauer and colleagues [13] demonstrated the competition of either crude marihuana extract (containing approximately 20 μM Δ^9-THC) or 6 μM CBD for binding of [^{3}H]estradiol to the estrogen receptor from rat uterine cytosol. However, in that same study, 70 μM Δ^9-THC or 20 μM concentrations of ten hydroxylated metabolites of Δ^9-THC failed to compete [13]. Thus, the data regarding binding of members of the cannabinoid class of compounds to the estrogen receptor are conflicting.

The functional significance of putative binding of cannabinoid compounds to estrogen receptors has yet to be defined. Inferential studies in intact animals have approached the question of whether Δ^9-THC behaves as an estrogen receptor agonist in studies of uterine growth, and results have again been conflicting [14–16]. However, interpretation of these studies is confounded by the actions of cannabinoid compounds at alternative sites in the hypothalamic-pituitary-gonadal axis. The true test of whether cannabinoid compounds exhibit agonist ability at estrogen receptors would be the direct demonstration of effects on transcription via the estrogen response element controlling gene expression. If Δ^9-THC and its analogs behave as agonists, then they should mimic the effects of estradiol on gene expression and the consequent response such as the proliferative response in the MCF-7 breast cancer cell model. An alternative hypothesis, that Δ^9-THC and analogs behave as estrogen receptor antagonists, would be demonstrated if these compounds blocked the transcription and proliferative responses to estradiol. In the present paper, these two alternatives were tested.

MATERIALS AND METHODS

Materials

MEM with nonessential amino acids, HEPES, bovine insulin, calf thymus DNA type I, Hoechst dye 33258, streptomycin sulfate and penicillin-G, and 17β-estradiol were obtained from the Sigma Chemical Co. (St. Louis, MO), "Cell Culture Tested" when available. LY156758 (keoxifene; raloxifene) was a gift from Eli Lilly & Co. (Indianapolis, IN). Bovine calf serum, lyophilized trypsin, and phenol red (sodium salt) were from Gibco BRL (Grand Island, NY). Formononetin was obtained from the Atomergic Chemicals Corp. (Farmingdale, NY). Δ^9-THC and CBD were provided by the National Institute on Drug Abuse, and DALN was a gift from Pfizer, Inc. (Groton, CT). All other chemicals were reagent grade.

Cell Culture

MCF-7 cells were obtained from Dr. V. C. Jordan, University of Wisconsin-Madison. MCF-7 cells were maintained in MEM containing nonessential amino acids, phenol red (10 μg/mL), 10 mM HEPES, insulin (6 ng/mL), penicillin (100 U/mL), streptomycin (100 μg/mL), and 5% charcoal-stripped bovine calf serum (maintenance medium) [17]. Because the responsiveness of MCF-7 cells maintained continuously in stripped calf serum can drift [18], cells were propagated in stripped calf serum for only a year or so before being discarded and replaced with cells derived from our primary source, MCF-7 cells that had been maintained in whole serum before storage in liquid N_2. Cells tested negative for mycoplasma before and after the course of the present experiments.

Cell Proliferation Assay

The proliferative response to estrogens was determined as previously described [18, 19]. Except where noted, 12,500 MCF-7 cells were seeded in 1 mL/well of a 24-well plate on Day 0 in estrogen-free (phenol red-free) maintenance medium. The cells were fed on Day 1 with the same medium, and treated for Days 3 through 6 with test medium containing compounds at the indicated concentrations, with daily medium changes. On Day 7, the wells were washed with 1 mL of HBSS. The washed cells were dissolved in 925 μL of 10 mM EDTA, pH 12.3 (37°, 20 min), neutralized with 75 μL of 0.77 M KH_2PO_4, and sonicated. Then aliquots were taken for measurements of DNA fluorometrically with Hoechst dye 33258 according to the method of Labarca and Paigen [20]. Calf thymus DNA was used as the standard after calibration by absorbance at 254 nm, assuming 20 absorbance units for 1 mg DNA/mL. Data were obtained as the mean μg DNA per well from triplicate determinations, and normalized such that the basal values were the minimal (0%) and the values at 100 pM 17β-estradiol were the maximal (100%) responses. For the experiments reported in this study, the basal values were 3.5 ± 0.37 μg DNA/well, and the response to 100 pM 17β-estradiol averaged 2.5 ± 0.12-fold greater than the basal values (mean ± SEM, N = 7).

Transient Transfections and CAT Assays

For the transcriptional activation studies, the estrogen-responsive plasmid ERE*tk*CAT was used (provided by Dr. Ming Tsai, Baylor College of Medicine, Houston, TX). ERE*tk*CAT plasmids were constructed by insertion of a 21-mer oligonucleotide containing vitellogenin A2 sequences from −332 to −318 and *Bgl*II overhangs into pBLCAT2 as described [21]. pBLCAT2 contains the promoter of the herpes simplex virus thymidine kinase (tk)

gene coupled to the CAT gene of *Escherichia coli* and RNA processing signals of simian virus 40. Transient transfections were performed by the calcium-phosphate coprecipitation method [22] with some modifications. MCF-7 cells were plated as described above at a density of 120,000 cells/9.6 cm^2 well of 6-well plates, in 1.2 mL of estrogen-free medium. After 4 days of growth and one medium change, the medium was removed and replaced with 1.0 mL fresh medium, and 200 μL of the DNA precipitates containing 4 μg reporter plasmid was then added to each well. After incubation at 37°C for 15 hr, the medium was removed, and cells were treated with various concentrations of the appropriate agents in estrogen-free medium for 48 hr. The CAT assays were performed as previously described [23]. CAT activity was calculated as the percentage of acetylated forms per total forms (nonacetylated chloramphenicol plus acetylated forms) and expressed as percent of the maximal estrogen-stimulated CAT activity.

RESULTS

Effects of Estrogens Versus Cannabinoids on Cell Proliferation

Estrogens stimulate the proliferation of the MCF-7 cells in culture, and this response is particularly evident when phenol red and steroids in the culture serum component are deleted from the growth medium. As shown in Fig. 1, 17β-estradiol at 0.1 pM was sufficient to promote proliferation, and a maximal stimulation was observed at 100 pM 17β-estradiol. Three cannabinoid compounds were tested for similar responses. Neither Δ^9-THC, DALN, nor CBD was

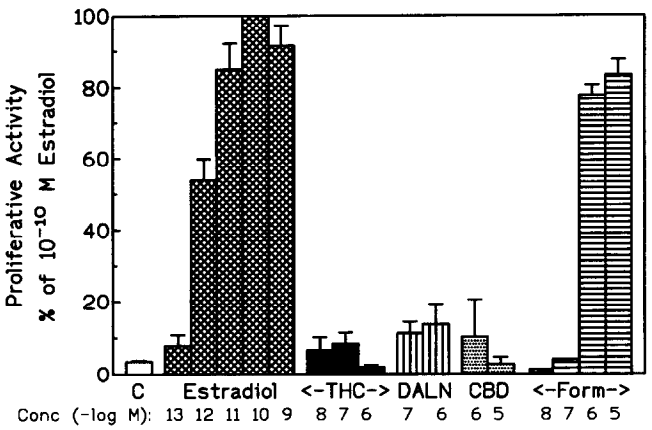

FIG. 1. Failure of cannabinoid compounds to elicit an estrogenic proliferative response. MCF-7 cells were treated with the indicated concentrations of compounds, and the data are expressed as the means ± SEM of normalized responses from 3 to 7 experiments. Data from multiple experiments were analyzed by ANOVA followed by Tukey's post-hoc test. Form = formononetin. Values for 10 nM to 1 μM Δ^9-THC, 0.1 and 1 μM DALN, and 1 and 10 μM CBD were not significantly different from each other and, with the exception of 1 μM DALN, were not significantly different from control ($P < 0.05$).

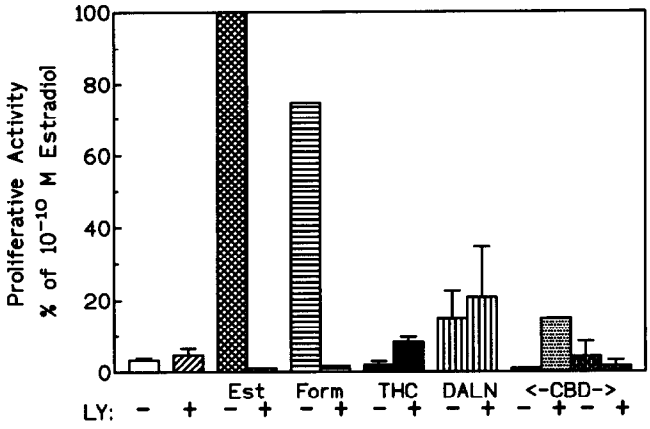

FIG. 2. Reversal of estrogen receptor-mediated responses by LY156758. MCF-7 cells were incubated with 100 pM 17β-estradiol (Est), 1 μM formononetin (Form), 1 μM Δ^9-THC, 1 μM DALN, and 1 or 10 μM CBD in the absence (-) or presence (+) of 100 nM LY156758 (LY) as described in the text. Data are the average responses from 2 or 3 experiments (±range) for the cannabinoid compounds and from a single representative experiment for the estrogens 17β-estradiol and formononetin.

able to stimulate the proliferative response to a significant extent above control. The phytoestrogen formononetin [24] was included in these studies as an example of an environmental "weak" estrogen. This isoflavone is one of the weaker phytoestrogens in the estrogen-stimulated proliferation bioassay [19]. Formononetin and its metabolites are responsible for the infertility syndrome "clover disease" in sheep grazing pastures rich in subterranean clover, *Trifolium subterraneum* [24, 25]. Although its potency is 4–5 orders of magnitude lower than 17β-estradiol, it nevertheless produces a response equal in efficacy to that of 17β-estradiol (Fig. 1).

Incubation of MCF-7 cells with the antiestrogen LY156758 [26] did not reduce the growth rate in the proliferation assay below that of the control (Fig. 2). This indicates that the estrogen-free medium used in these experiments was indeed free of any physiological levels of estrogens. This compound was an effective antagonist of the responses to 17β-estradiol and formononetin, and yet it had no influence on the failure of the cells to respond to cannabinoid compounds (Fig. 2). LY156758 was used because it exhibits less partial estrogen agonist activity than does tamoxifen, yet does not lead to rapid loss of estrogen receptors as has been reported for the pure estrogen antagonist ICI164384 [27].

To determine if cannabinoid compounds might act as antagonists at the estrogen receptor, these compounds were incubated with MCF-7 cells in the presence of a concentration of 17β-estradiol that produced a submaximal response (Fig. 3). Neither Δ^9-THC nor DALN at concentrations of 1 μM (Fig. 3) or 10 μM Δ^9-THC (data not shown) was able to inhibit the estrogenic stimulation of the proliferative response under conditions in which 100 nM

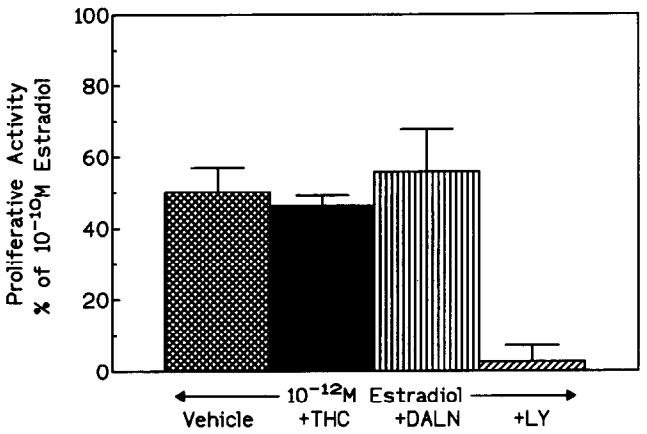

FIG. 3. Failure of cannabimimetic compounds to behave as estrogen receptor antagonists. MCF-7 cells were incubated with 1 pM 17β-estradiol in the absence or presence of 1 μM Δ^9-THC, 1 μM DALN, or 100 nM LY156758. Cannabinoid data (means ± SEM of responses from 3 experiments normalized to 100 pM 17β-estradiol) analyzed by ANOVA resulted in no significant differences from the estrogen control.

LY156758 was completely effective as an antagonist. CBD at 1 or 10 μM was unable to inhibit the response to 1 pM 17β-estradiol in two separate experiments (data not shown). The 10 μM concentration of CBD resulted in cytotoxicity in two other experiments, and thus interpretation of data based upon cell proliferation at this concentration of drug is not possible.

It was noted in these experiments that 10 μM concentrations of DALN exerted a profound toxic effect on the cells, inhibiting their proliferation below the basal rate of cell growth. CBD at 10 μM reduced cell proliferation below that of the basal rate in two out of four experiments; however, the extent of the cell loss was not as great as for DALN. The cytoxicity was not shared by Δ^9-THC, although the latter compound was not tested at concentrations greater than 10 μM to determine if higher concentrations would alter cell proliferation in a negative way. This toxicity was unexpected inasmuch as exposure of N18TG2 neuroblastoma cells to 10–100 μM DALN (or Δ^9-THC) for up to 48 hr failed to compromise their growth rate, plating efficiency, or morphology as determined by scanning or transmission electron microscopy [28]. However, cytotoxicity has been reported previously for Δ^9-THC at concentrations that exceed 10 μM in other cell types [29–32]. The toxicity to 10 μM DALN was not related to the estrogen receptor because it was not altered by LY156758. Furthermore, cells grown in 10 μM DALN failed to survive even in the presence of 1 pM or 1 nM 17β-estradiol. It is recognized that Δ^9-THC is not water soluble at concentrations that exceed 1–10 μM in the temperature range and salt concentrations in the medium utilized in the present study [32]. Thus, partitioning into the membranes of cells is not unexpected for cannabinoid compounds at this high concentration [33–35].

Effects on Transcriptional Activation: Estrogens Versus Cannabinoids

The stimulation of the estrogen receptor by agonists initiates a transcriptional stimulation via the ERE in target genes. MCF-7 cells transfected with the EREtkCAT reporter gene and treated with 1 pM to 100 nM 17β-estradiol demonstrated a concentration-dependent increase in transcriptional activation with a maximum 13-fold increase in CAT activity at concentrations above 100 pM (Figs. 4 and 5). The antiestrogen LY156758, which has been reported to show only slight partial agonist activity, revealed a very modest (1.8-fold) increase over basal CAT activity at 100 nM which was not statistically significant. The phytoestrogen formononetin demonstrated a 3.4- and 6.7-fold increase over basal CAT activity with 1 and 10 μM, respectively (Fig. 5B). Neither Δ^9-THC, DALN, nor CBD was able to stimulate transcriptional activity. Thus, the cannabinoid compounds tested cannot be considered estrogens in a well-established assay of estrogenic activity.

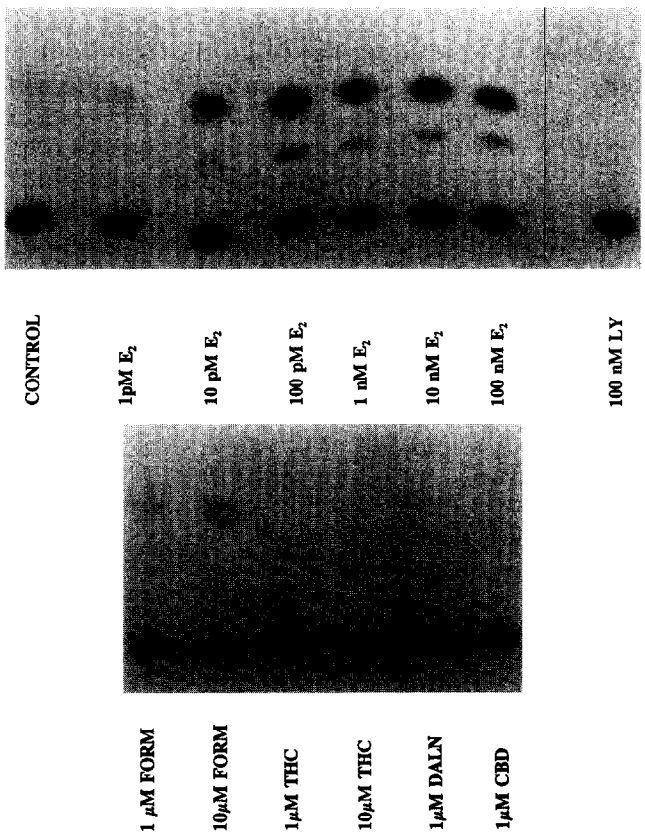

FIG. 4. Stimulation of transcription in a transfected EREtkCAT expression system by estrogens. Treatment of MCF-7 cells with 17β-estradiol (E_2) or LY156758 (LY) (top), or formononetin (FORM) or the cannabinoid compounds (bottom) at the indicated concentrations and assay of CAT activity are as described in the text. Shown are autoradiograms of the thin-layer chromatography separations of chloramphenicol from acetylated metabolites.

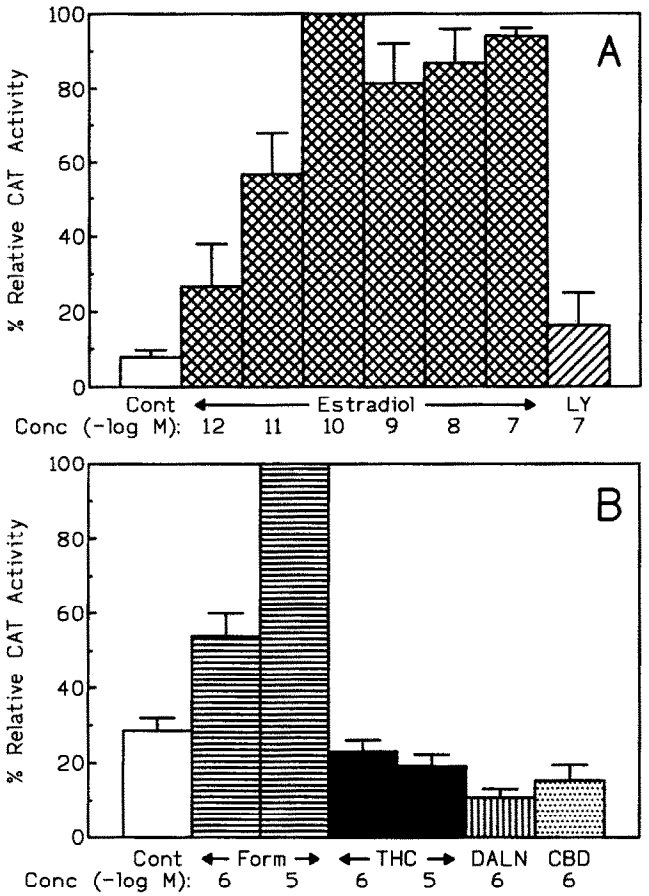

FIG. 5. CAT activity expressed in response to estrogens but not cannabinoid compounds. (A) Normal estrogenic transcriptional response. CAT values have been normalized such that the response to 100 pM 17β-estradiol is expressed as 100%, and stimulation by 1 pM to 100 nM 17β-estradiol or 100 nM LY156758 (LY) is relative to that value. (B) Transcriptional response to formononetin (Form), Δ^9-THC, DALN, or CBD at the indicated concentrations. CAT values have been normalized such that the maximal response to 10 μM formononetin (Form) is shown as 100%. This stimulation by formononetin represents 65% of the maximal response to 100 pM 17β-estradiol. Data shown are the means ± SEM from 3 wells.

DISCUSSION

Considerable evidence exists to link cannabinoid drugs to endocrine physiology, and particularly to reproductive physiology. Clearly, many effects are attributable to cannabinoceptive neurons in brain pathways that ultimately regulate pituitary function (for review, see Refs. 2 and 3). However, because both cannabinoid and steroid molecules are multi-ringed structures possessing hydroxyl groups, it is not illogical to hypothesize that the cannabinoid class of compounds might associate with estrogen receptors, and vice versa. The present study specifically addressed the question of whether cannabinoid compounds would result in a functional interaction with estrogen receptors. The cannabinoid compounds chosen include Δ^9-THC, the primary active cannabimimetic agent in *Cannabis* preparations, and DALN, a tricyclic cannabinoid compound having a structure homologous to that of Δ^9-THC but with much greater potency to stimulate cannabinoid receptors [36, 37]. CBD was also tested because it is found in abundance in preparations of *Cannabis* and has been reported to have some biological actions although it has limited ability to promote a cannabimimetic response [38–40], and it has been implicated as an agent that might interact with the estrogen receptor [13]. Using two sensitive and well-established tests for estrogen receptor activation, the evidence fails to support the hypothesis that cannabinoid compounds can behave as agonists at estrogen receptors. The notion that cannabinoid compounds including Δ^9-THC are environmental phytoestrogens (see, for example, Ref. 41) cannot be supported by the data presented here. Phytoestrogens, including flavonoids, have been found to bind to the estrogen receptor over a wide range of potencies, and to produce a wide range of estrogenic efficacies [19, 42, 43]. Certain bioflavonoid compounds have been shown to exhibit antiestrogenic activity [44]. However, the present data indicate that cannabinoid compounds cannot be considered as potential environmental estrogens.

A potential for interaction between cannabinoid compounds and the steroid receptors for glucocorticoids has also been investigated. One study that examined [^{3}H]Δ^8-THC binding in crude nuclear fractions from hepatoma tissue culture cells found that only a small fraction of the bound [^{3}H]Δ^8-THC could be displaced by dexamethasone, arguing against the binding of the cannabinoid to hepatic glucocorticoid receptors [45]. The converse experiment, which showed a partial displacement of [^{3}H]dexamethasone by Δ^9-THC or CBD in rat hippocampal cytosol, suggested that some affinity of cannabinoid compounds exists for the brain glucocorticord receptors [46, 47]. Functional studies to demonstrate an activation of the glucocorticoid response element by cannabinoid compounds have not been reported.

When steroid ligands, including 17β-estradiol, progesterone, pregnenolone sulfate, androsterone, cortisone, and corticosterone, were tested for their ability to displace the potent agonist ligands [^{3}H]CP-55940 or [^{3}H]WIN55212-2 from CB_1 cannabinoid receptors in rat brain membranes, no interaction was noted at concentrations as high as 1 or 10 μM [48–50]. Thus, it is not likely that steroid compounds present at their physiological free concentrations in biological fluids would bind to cannabinoid receptors in the brain. These data together with the findings in the present report negate the hypothesis that there exist some affinity of cannabinoid compounds for estrogen receptors and the converse.

Current efforts to identify and investigate environmental factors, including phytoestrogens, that functionally mimic estradiol have resulted in a growing list of diverse chemicals that bind to the estrogen receptor and may result in altered reproductive function and/or increased risk for breast can-

cer. However, functional analysis of purported estrogenic compounds in well-defined systems is necessary to define a compound as an estrogen, i.e. as a compound that can activate the estrogen receptor. Our studies are the first to directly study the cannabinoid class of compounds in functional assays for estrogenic activity. The results presented here fail to support the notion that cannabinoid compounds are xenoestrogens.

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References

1. Smith CG and Asch RH, Drug abuse and reproduction. *Fertil Steril* **48:** 355–373, 1987.
2. Murphy LL, Steger RW and Bartke A, Psychoactive and nonpsychoactive cannabinoids and their effects on reproductive neuroendocrine parameters. In: *Biochemistry and Physiology of Substance Abuse* (Ed. Watson RR), pp. 73–93. CRC Press, Boca Raton, 1990.
3. Wenger T, Croix D, Tramu G and Leonardelli J, Effects of Δ^9-tetrahydrocannabinol on pregnancy, puberty, and the neuroendocrine system. In: *Marijuana/Cannabinoids. Neurobiology and Neurophysiology* (Eds. Murphy L and Bartke A), pp. 539–560. CRC Press, Boca Raton, 1992.
4. Gerard CM, Mollereau C, Vassart G and Parmentier M, Molecular cloning of a human cannabinoid receptor which is also expressed in testis. *Biochem J* **279:** 129–134, 1991.
5. Das SK, Paria BC, Chakraborty I and Dey SK, Cannabinoid ligand–receptor signaling in the mouse uterus. *Proc Natl Acad Sci USA* **92:** 4332–4336, 1995.
6. Galiegue S, Sophie M, Marchand J, Dussossoy D, Carriere D, Carayon P, Bouaboula M, Shire D, LeFur G and Casellas P, Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem* **232:** 54–61, 1995.
7. Abood ME and Martin BR, Neurobiology of marijuana abuse. *Trends Pharmacol Sci* **13:** 201–206, 1992.
8. Howlett AC, Cannabinoid compounds and signal transduction mechanisms. In: *Cannabinoid Receptors: Molecular Biology and Pharmacology* (Ed. Pertwee RG), pp. 167–204. Academic Press, London, 1995.
9. Howlett AC, Pharmacology of cannabinoid receptors. *Annu Rev Pharmacol Toxicol* **35:** 607–634, 1995.
10. Rawitch AB, Schultz GS, Ebner KE and Vardaris RM, Competition of Δ^9-tetrahydrocannabinol with estrogen in rat uterine estrogen receptor binding. *Science* **197:** 1189–1191, 1977.
11. Okey AB and Bondy GP, Is delta-9-tetrahydrocannabinol estrogenic? *Science* **195:** 904–906, 1977.
12. Chakravarty I and Naik VK, Action of delta-9-tetrahydrocannabinol on the binding of estradiol to uterine and other tissues in rats. *Biochem Pharmacol* **32:** 253–256, 1983.
13. Sauer MA, Rifka SM, Hawks RL, Cutler GB Jr and Loriaux DL, Marijuana: Interaction with the estrogen receptor. *J Pharmacol Exp Ther* **224:** 404–407, 1983.
14. Okey AB and Truant GS, Cannabis demasculinizes rats but is not estrogenic. *Life Sci* **17:** 1113–1118, 1975.
15. Solomon J, Cocchia MA, Gray R, Shattuck D and Vossmer A, Uterotrophic effect of delta-9-tetrahydrocannabinol in ovariectomized rats. *Science* **192:** 559–561, 1976.
16. Virgo BB, The estrogenicity of delta-9-tetrahydrocannabinol (THC): THC neither blocks nor induces ovum implantation, nor does it effect uterine growth. *Res Commun Chem Pathol Pharmacol* **25:** 65–77, 1979.
17. Grady LH, Nonneman DJ, Rottinghaus GE and Welshons WV, pH-Dependent cytotoxicity of contaminants of phenol red for MCF-7 breast cancer cells. *Endocrinology* **129:** 3321–3330, 1991.
18. Read LD, Greene GL and Katzenellenbogen BS, Regulation of estrogen receptor messenger ribonucleic acid and protein levels in human breast cancer cell lines by sex steroid hormones, their antagonists, and growth factors. *Mol Endocrinol* **3:** 295–304, 1989.
19. Welshons WV, Rottinghaus GE, Nonneman DJ, Dolan-Timpe M and Ross PF, A sensitive bioassay for detection of dietary estrogens in animal feeds. *J Vet Diag Invest* **2:** 268–273, 1990.
20. Labarca C and Paigen K, A simple, rapid and sensitive DNA assay procedure. *Anal Biochem* **102:** 344–352, 1980.
21. Klein-Hitpass L, Tsai SY, Greene GL, Clark JH, Tsai M-J and O'Malley BW, Specific binding of estrogen receptor to the estrogen response element. *Mol Cell Biol* **9:** 43–49, 1989.
22. Chen C and Okayama H, High efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* **7:** 2745–2752, 1987.
23. Gorman CM, Moffat LF and Howard BH, Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol Cell Biol* **2:** 1044–1051, 1982.
24. Shutt DA, The effects of plant oestrogens on animal reproduction. *Endeavour* **35:** 110–113, 1976.
25. Bennets HW, Underwood EJ and Shier FL, A specific breeding problem of sheep on subterranean clover pastures in Western Australia. *Aust Vet J* **22:** 2–12, 1946.
26. Black LJ, Jones CD and Falcone JF, Antagonism of estrogen action with a new benzothiophene-derived antiestrogen. *Life Sci* **32:** 1031–1036, 1983.
27. Gibson MK, Nemmers LA, Beckman WCJ, Davis VL, Curtis SW and Korach KS, The mechanism of ICI164,384 antiestrogenicity involves rapid loss of estrogen receptor in uterine tissue. *Endocrinology* **129:** 2000–2010, 1991.
28. Dill JA and Howlett AC, Regulation of adenylate cyclase by chronic exposure to cannabimimetic drugs. *J Pharmacol Exp Ther* **244:** 1157–1163, 1988.
29. Raz A and Goldman R, Effect of hashish compounds on mouse peritoneal macrophages. *Lab Invest* **34:** 69–76, 1976.
30. Kelly LA and Butcher RW, Effects of Δ^1-tetrahydrocannabinol on cyclic AMP in cultured human diploid fibroblasts. *J Cyclic Nucleotide Res* **5:** 303–313, 1979.
31. Cooper JT and Goldstein S, Toxicity testing *in vitro*. I. The effects of Δ^9-tetrahydrocannabinol and aflatoxin B_1 on the growth of cultured human fibroblasts. *Can J Physiol Pharmacol* **54:** 541–545, 1976.
32. Garrett ER and Hunt CA, Physiochemical properties, solubility, and protein binding of Δ^9-tetrahydrocannabinol. *J Pharm Sci* **63:** 1056–1064, 1974.
33. Seeman P, Chau-Wong M and Moyyen S, The membrane binding of morphine, diphenylhydantoin, and tetrahydrocannabinol. *Can J Physiol Pharmacol* **50:** 1193–1200, 1972.
34. Colburn RW, Ng LK, Lemberger L and Kopin IJ, Subcellular distribution of Δ^9-tetrahydrocannabinol in rat brain. *Biochem Pharmacol* **23:** 873–877, 1974.
35. Roth SH and Williams PJ, The non-specific membrane binding properties of Δ^9-tetrahydrocannabinol and the effects of various solubilizers. *J Pharm Pharmacol* **31:** 224–230, 1979.
36. McIlhenny HM, Mast RW, Johnson MR and Milne GM, Nantradol hydrochloride: Pharmacokinetics and behavioral effects after acute and chronic treatment. *J Pharmacol Exp Ther* **219:** 363–369, 1981.

37. Howlett AC, Johnson MR, Melvin LS and Milne GM, Nonclassical cannabinoid analgetics inhibit adenylate cyclase: Development of a cannabinoid receptor model. *Mol Pharmacol* **33:** 297–302, 1988.
38. Carlini EA and Cunha JM, Hypnotic and antiepileptic effects of cannabidiol. *J Clin Pharmacol* **21:** Suppl 417S–427S, 1981.
39. Hollister LE, Cannabidiol and cannabinol in man. *Experientia* **29:** 825–826, 1973.
40. Perez-Reyes M, Timmons MC, Davis KH and Wall EM, A comparison of the pharmacological activity in man of intravenously administered Δ^9-tetrahydrocannabinol, cannabinol, and cannabidiol. *Experientia* **29:** 1368–1369, 1973.
41. Raloff J, Ecocancers. Do environmental factors underlie a breast cancer epidemic? *Sci News* **144:** 10–13, 1993.
42. Miksicek RJ, Commonly occurring plant flavonoids have estrogenic activity. *J Pharmacol Exp Ther* **44:** 37–43, 1993.
43. Miksicek RJ, Estrogenic flavonoids: Structural requirements for biological activity. *Proc Soc Exp Biol Med* **208:** 44–50, 1995.
44. Ruh MF, Zacharewski T, Connor K, Howell J, Chen I and Safe S, Naringenin: A weakly estrogenic bioflavonoid that exhibits antiestrogenic activity. *Biochem Pharmacol* **50:** 1485–1493, 1995.
45. Harris LS, Carchman RA and Martin BR, Evidence for the existence of specific cannabinoid binding sites. *Life Sci* **22:** 1131–1137, 1978.
46. Eldridge JC and Landfield PW, Cannabinoid interactions with glucocorticoid receptors in rat hippocampus. *Brain Res* **534:** 135–141, 1990.
47. Eldridge JC, Murphy LL and Landfield PW, Cannabinoids and the hippocampal glucocorticoid receptor: Recent findings and possible significance. *Steroids* **56:** 226–231, 1991.
48. Bidaut-Russell M, Devane WA and Howlett AC, Cannabinoid receptors and modulation of cyclic AMP accumulation in the rat brain. *J Neurochem* **55:** 21–26, 1990.
49. Howlett AC, Evans DM and Houston DB, The cannabinoid receptor. In: *Marijuana/Cannabinoids: Neurobiology and Neurophysiology* (Eds. Murphy L and Bartke A), pp. 35–72. CRC Press, Boca Raton, 1992.
50. Kuster JE, Stevenson JI, Ward SJ, D'Ambra TE and Haycock DA, Aminoalkylindole binding in rat cerebellum: Selective displacement by natural and synthetic cannabinoids. *J Pharmacol Exp Ther* **264:** 1352–1363, 1993.