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Dual suppression of estrogenic and inflammatory activities for targeting of endometriosis

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SUPPLEMENTARY MATERIALS

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Fig. S2. Effect of OBHS or CLI on endometriotic lesion establishment and eutopic uterine weight.

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Fig. S5. Effects of OBHS and CLI on mammary gland.

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Abstract

Estrogenic and inflammatory components play key roles in a broad range of diseases including endometriosis, a common estrogen-dependent gynecological disorder in which endometrial tissue creates inflammatory lesions at extrauterine sites, causing pelvic pain and reduced fertility. Current medical therapies focus primarily on reducing systemic levels of estrogens, but these are of limited effectiveness and have considerable side effects. We developed estrogen receptor (ER) ligands, chloroindazole (CLI) and oxabicycloheptene sulfonate (OBHS), which showed strong ER-dependent anti-inflammatory activity in a preclinical model of endometriosis that recapitulates the estrogen dependence and inflammatory responses of the disease in immunocompetent mice and in primary human endometriotic stromal cells in culture. Estrogen-dependent phenomena, including cell proliferation, cyst formation, vascularization, and lesion growth, were all arrested by CLI or OBHS, which prevented lesion expansion and also elicited regression of established lesions, suppressed inflammation, angiogenesis, and neurogenesis in the lesions, and interrupted crosstalk between lesion cells and infiltrating macrophages. Studies in ER α or ER β knockout mice indicated that ER α is the major mediator of OBHS effectiveness and ER β is dominant in CLI actions, implying involvement of both ERs in endometriosis. Neither ligand altered estrous cycling or fertility at doses that were effective for suppression of endometriosis. Hence, CLI and OBHS are able to restrain endometriosis by dual suppression of the estrogen-inflammatory axis. Our findings suggest that these compounds have the desired characteristics of preventive and therapeutic agents for clinical endometriosis and possibly other estrogen-driven and inflammation-promoted disorders.

INTRODUCTION

Pathological effects of estrogens in disorders of reproductive and other target tissues can be exacerbated by an inflammatory environment, the origin of which is not always clear. Although there are well-established means for suppressing or moderating estrogenic drive, it is less clear how the inflammatory component might best be managed. Endometriosis is a paradigmatic estrogen-dependent, inflammatory disorder defined by the attachment of endometrial tissue at extrauterine ectopic sites where it forms inflammatory, invasive lesions (1–4). The urgent need to better understand the mechanisms underlying endometriosis, to enable development of more effective treatments, is driven by the fact that endometriosis affects 10 to 14% of reproductive-age women and 35 to 50% of those with pelvic pain and infertility (3), with annual costs exceeding \$20 billion in the United States alone (1).

The most effective medical treatments, such as progestins, androgens, gonadotropin-releasing hormone (GnRH) agonists, and aromatase inhibitors, focus on reducing systemic levels of estrogens. Unfortunately, these treatments are associated with untoward side effects and are not fully effective, and disease recurrence is frequent (2). Although the basis for endometriosis-associated pain is not fully understood, studies in women and animal models suggest that it might involve a coordinated program of neuronal and vascular infiltration of endometriotic tissue, termed neuroangiogenesis (1, 5). In addition, a high correlation of pain symptoms and inflammation has been noted clinically (6).

The estrogen dependence of endometriosis is well established (2, 3). It includes overexpression of the aromatase gene CYP19A1, responsible for local estrogen synthesis, and overexpression or increased activity of estrogen receptors (ERs) that elicit hyperestrogenic stimulation in lesions (7) and appear to be the drivers of disease progression (8). During disease pathogenesis, hyperestrogenic stimulation and inflammation are linked by a feed-forward loop sustained by overexpression of cyclooxygenase 2 (COX2) and CYP19A1, causing continuous local production of prostaglandins and estrogen (2). A highly activated nuclear factor κ B (NF κ B) pathway also contributes to this inflammatory state by stimulating expression of proinflammatory cytokines and chemokines (9). Because excessive estrogen stimulation and enhanced inflammation are pivotal aspects of endometriosis, we hypothesized that effective treatments should aim to suppress both of these components, as well as downstream mediators of neuroangiogenesis that may be effectors of pain.

Recently, we developed two ER ligands, chloroindazole (CLI) and oxabicycloheptene sulfonate (OBHS), with CLI exhibiting ER β -dependent activity and OBHS displaying more ER α -preferential binding selectivity, and both ligands optimized for having strong anti-inflammatory activity (10–13). Here, we have evaluated the efficacy of OBHS and CLI in treating endometriosis in a validated murine model in which endometriosis-like lesion establishment and progression are estrogen-dependent, similar to the clinical syndrome (14, 15). This model involves mice with a fully intact immune system, enabling us to evaluate the contribution of immune cells in lesion establishment and progression and accurately quantify the size of the lesions. Our results highlight the efficacy of OBHS and CLI in preventing the establishment of endometriotic lesions and in reversing the growth and progression of established lesions by targeting both estrogen-regulated and inflammatory activities, despite having minimal effects on reproductive physiology and fertility of host animals.

RESULTS

OBHS and CLI prevent ectopic lesion establishment

We first used a prevention model to examine the effectiveness of OBHS and CLI in preventing ectopic lesion establishment in mature female mice (Fig. 1A). Donor uterine fragments were engrafted onto the peritoneal wall of ovariectomized recipient mice treated with estradiol (E₂), where they formed endometriotic-like lesions. Over the 14-day study, ectopic lesions became about threefold larger in E₂-treated hosts, but this E₂-stimulated growth was fully blocked by cotreatment with OBHS or CLI (Fig. 1, B and C). Hematoxylin and eosin (H&E) staining (Fig. 1D) revealed that E₂ supported formation of cystic endometriotic lesions, a hallmark feature of human endometriosis; these were suppressed with OBHS or CLI. Figure S1 shows that substantial OBHS and CLI blood levels were maintained over 14 days by ligand pellet delivery.

Because estrogen-supported cell proliferation and neovascularization are required for establishment of endometriosis, we investigated the impact of OBHS and CLI on these two aspects. OBHS or CLI treatment significantly reduced Ki67 ($P = 0.02$), a marker for E₂-stimulated proliferation, in the ectopic lesions (Fig. 1, E and F). Similarly,

immunofluorescence for the endothelial cell marker platelet endothelial cell adhesion molecule (PECAM) (Fig. 1G) and quantification of microvessel density (Fig. 1H) demonstrated that E₂-supported vascularization was severely impaired. Moreover, E₂-induced increase in cysteine-rich protein 61 (*CYR61*) and vascular endothelial growth factor A (*VEGFA*), two potent proangiogenic factors, was fully blocked by OBHS or CLI at both mRNA (Fig. 1I) and protein levels (Fig. 1J). Collectively, these results indicate that OBHS and CLI antagonize multiple aspects of E₂ activity and are able to interrupt ectopic lesion establishment.

When OBHS or CLI was given alone to ovariectomized recipients, lesion volume was reduced to below that of vehicle (fig. S2A), suggesting that actions of estrogen produced locally by aromatase in ectopic lesions (7, 16) are being suppressed by the compounds. OBHS or CLI given alone had no stimulatory effects on the weight of the recipient mouse uterus (fig. S2B). As expected, both compounds were able to greatly suppress E₂-stimulated eutopic uterine tissue growth and cell proliferation (fig. S3, A to C), consistent with their antiestrogenic activity.

OBHS and CLI reverse established lesions with little effect on host fertility

We next determined the efficacies of OBHS and CLI in treating established lesions using a therapeutic model, in which ectopic tissues were allowed to become established in intact recipients for 2 weeks before compounds were administered (Fig. 2A). Assessments were done after 2 or 6 weeks of compound treatment. At the end of 6 weeks of a dose-response study, the 0.25 mg per pellet dose markedly reduced lesion size in these recipients with intact ovarian function (Fig. 2B, left), with little or no impact on host uterine weight (Fig. 2B, right); thus, this dose was selected for further studies. Ectopic lesions kept growing in intact recipient mice with control vehicle treatment, whereas ectopic tissues in treated animals failed to grow, even regressing over the 6-week treatment (Fig. 2C). Consistent with observations in the prevention model (Fig. 1, F to I), cell proliferation (Fig. 2, D and E) and neovascularization in ectopic lesions (Fig. 2, F and G) were also markedly suppressed by ligand treatment.

No statistically significant changes were detected in host eutopic uterine weight (fig. S4A), and Ki67 staining showed that eutopic uterine cell proliferation was unchanged with either ligand (fig. S4, B and C). In addition, at the 0.25 mg per pellet dose, no difference was observed in mammary gland histology (fig. S5, A and B) in mice treated with ligands or vehicle control. Ovarian histology (fig. S6A) and examination of the length of each estrous cycle stage (fig. S6B) also suggested maintenance of normal ovarian function in recipient mice over the 6-week ligand treatment. Fertility of host females was also not delayed or reduced by prior ligand treatment, on the basis of a 4-month breeding study in which the numbers of litters and pups per litter were similar for ligand- and vehicle-treated animals; the pups in all groups appeared healthy and showed similar body weights measured at weaning (21 days; table S1).

OBHS and CLI have anti-inflammatory effects during lesion progression

In the therapeutic model, we profiled the mRNA expression levels of several cytokines reported to be highly expressed in human endometriosis (Fig. 3A) (17–19). Decreased mRNA levels of interleukin-6 (*Il6*), chemokine (C-C motif) ligand 2 (*Ccl2*), 5 (*Ccl5*), and tumor necrosis factor α (*Tnfa*) were observed in ectopic lesions over a 6-week period of ligand treatment. Immunostaining also documented reduced levels of IL-6 protein in OBHS- or CLI-treated lesions (Fig. 3, B and C), supporting the anti-inflammatory effects of the ligands. Another hallmark of endometriosis-associated inflammation is NF κ B activation (20). NF κ B activity observed by nuclear staining of its subunit p65 was greatly reduced in cells from OBHS- or CLI-treated ectopic lesions (Fig. 3, D and E). Likewise, the presence of the inflammation-associated protein COX2 was almost completely eliminated in ectopic lesions treated with OBHS or CLI (Fig. 3F). An increased number of apoptotic cells was detected in ligand-treated lesions by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assays (Fig. 3G).

To further characterize inflammation in ectopic lesions, we examined immune cell infiltration by immunofluorescence for the T cell marker CD3 and macrophage marker F4/80. To identify the origin of immune cells present in lesions, we double-stained sections from wild-type ectopic tissues transplanted into enhanced green fluorescent protein (EGFP) recipient mice with green fluorescent protein (GFP) and immune cell markers. As shown in fig. S7 (A and B), T cells and macrophages in the lesions labeled with CD3 or F4/80, respectively, were also found to be GFP-positive, confirming the recruitment of these cells from the host. The number of infiltrating immune cells was greatly reduced upon ligand treatment (Fig. 3H) ($P = 0.012$). These results collectively demonstrate that OBHS and CLI display potent anti-inflammatory activities during lesion progression, suppressing key cytokines, chemokines, and cellular immune responses characteristic of endometriosis.

OBHS and CLI reduce innervation and neuron activation in ectopic lesions

Endometriotic lesions are heavily innervated (21, 22), and studies in women and rats suggest that innervation is involved in endometriosis-associated pain (5, 21, 23, 24). Dual staining with GFP and a pan-neuron marker, protein gene product 9.5 (PGP 9.5), indicated that EGFP host-derived neurons were present in wild-type lesions. In addition, newly formed blood vessels with GFP-positive endothelial cells were closely associated with the infiltrating neurons in the lesions (fig. S8), supporting the hypothesis of coordinated neuroangiogenesis in endometriosis lesions.

To characterize the neuron phenotypes in the ectopic lesions, we performed dual staining for PGP 9.5 and substance P (SP) or calcitonin gene-related peptide (CGRP), which serve as neurotransmitter peptides and sensory nerve markers (22) (Fig. 4, A and B). The colocalization of PGP 9.5/SP and PGP 9.5/CGRP confirmed that sensory neurons are attracted into ectopic lesions. The classic nerve growth factor (NGF) and its receptor (NGFR) were also expressed in the ectopic lesions (Fig. 4C). The density of neurons, including sensory nerves, as well as NGF and NGFR, was strikingly reduced in ectopic lesions after 2 weeks of OBHS or CLI treatment (Fig. 4, A to C). Prostaglandins (25) and

NGF (23, 26) can activate or sensitize nerves, but both OBHS and CLI down-regulate the rate-limiting enzyme for prostaglandin synthesis, COX2, and NGF (Figs. 3F and 4C).

The therapeutic efficacy of letrozole is enhanced in combination with OBHS and CLI

On the basis of the reported overexpression of aromatase in ectopic lesions (2, 7, 27), aromatase inhibitors, such as letrozole, have been studied in endometriosis clinical trials (7, 28, 29). In our mouse model, we observed an about 15-fold elevation of *Cyp19a1* (aromatase) mRNA in lesions at 7 days after tissue implantation, and this elevation was maintained at 14 and 28 days (fig. S9). In our therapeutic model, we observed that growth of established lesions was reduced by 2 weeks of letrozole treatment, and somewhat more by treatment with OBHS or CLI alone (Fig. 5A). Cotreatment with letrozole plus OBHS or CLI caused a greater lesion regression (Fig. 5A). In contrast, although eutopic uterine tissue weight was slightly reduced with letrozole treatment alone, OBHS or CLI alone did not reduce uterine weight (Fig. 5B). No further change in weight or cell proliferation was seen in normal uterine tissue upon cotreatment with letrozole and OBHS or CLI, whereas ectopic lesions showed greater reduced cell proliferation with cotreatment (Fig. 5, B and C). Inflammatory cytokine production from ectopic lesions was reduced with letrozole treatment alone, as seen by lower levels of *Il6*, *Ccl2*, and *Ccl5* transcripts, and these levels were reduced much further by cotreatment with OBHS or CLI (Fig. 5D). Likewise, cotreatment with letrozole plus OBHS or CLI elicited greater suppression of lesion innervation and vascularization than with letrozole alone (Fig. 5E).

OBHS and CLI suppress human endometriotic stromal cell viability

To examine the effects of OBHS and CLI in human endometriotic cells, we used primary human endometriotic stromal cells (hESCs) derived from ovarian endometriomas, a common site of endometriosis in humans. Although different lesion types are recognized, isolated endometrioma cells have been the most extensively characterized for in vitro analysis, particularly for their ER status and cytokine production (10, 22). These were cultured and exposed to TNF α and E₂ to mimic the in vivo hyperestrogenic and inflammatory microenvironment. As shown in Fig. 6A, the proliferative effect of TNF α and E₂ in hESCs was blocked by cotreatment with OBHS or CLI. Consistent with this, immunofluorescence of phosphorylated histone 3, a mitotic phase marker, also revealed that TNF α - and E₂-increased mitotic activity was effectively suppressed by OBHS or CLI (Fig. 6, B and C). Moreover, TUNEL assays revealed a fourfold increase in apoptosis in hESCs treated with OBHS or CLI (Fig. 6, D and E).

In hESCs, OBHS or CLI also reduced cytokine mRNA production by E₂ plus TNF α , and this suppression was reversed by the antiestrogen fulvestrant (ICI182,780, a selective ER down-regulator) (Fig. 6F), indicating requirement for ER in the anti-inflammatory effects of our ligands. Furthermore, when supplemented with conditioned medium from OBHS- or CLI-treated hESCs, differentiated THP-1 macrophage-like M0 cells expressed diminished levels of transcripts for inflammatory factors and growth factors (fig. S10), highlighting the suppressive effects of our ligands on crosstalk between hESCs and immune cells.

Infiltrating macrophages are important for the preventive effects of CLI and OBHS against lesion establishment

To address the impact of our ligands on the immune system of host animals, infiltrating macrophages with both M1 and M2 phenotypes were first shown to be present in endometriotic lesions, and their presence was completely prevented by treatment with OBHS and CLI (fig. S11). To examine further the importance of macrophages in the preventive actions of OBHS and CLI, we depleted macrophages from the host animals by treatment with clodronate-containing liposomes (Fig. 7A); depletion was confirmed by F4/80 staining of ectopic lesions (Fig. 7B). Reduction of E₂-supported lesion growth was observed upon macrophage depletion, and both ligands partially lost their beneficial effects in preventing lesion growth (Fig. 7C) and cell proliferation (fig. S12), supporting a role for infiltrating macrophages in contributing to the growth of ectopic lesions. OBHS and CLI still suppressed E₂-enhanced lesion growth, but not as fully as observed in animals without clodronate treatment (Fig. 7C). Cytokine mRNA levels were greatly reduced in ectopic tissues upon macrophage depletion, and under these conditions, the ligands did not further suppress this very low cytokine production (Fig. 7D), indicating a critical contribution of the host immune response to the effectiveness of our ligands in preventing lesion establishment. Of interest, in eutopic uterus, clodronate treatment and macrophage depletion from host animals had no effect on uterine weight gain in response to E₂, implying that although macrophages support the growth of endometriotic lesions, they are not required for the E₂-stimulated proliferation of normal host uterine tissue (Fig. 7E).

ER α and ER β are involved in suppression of lesion progression by CLI and OBHS

Because our findings highlighted the multicellular nature of the endometriotic lesions, and ER α and ER β are present in many of these cells, we evaluated the importance of the two ER subtypes in host tissues and infiltrating immune cells for the suppressive effects of OBHS and CLI on endometriotic lesions. Ligand effectiveness was examined in wild-type, ER α knockout (α KO), and ER β knockout (β KO) mice. Wild-type donor tissues were transplanted into wild-type, α KO, or β KO intact mature recipient mice, and lesions were allowed to establish for 2 weeks, followed by 2 weeks of ligand treatment (therapeutic model).

Deletion of ER α in recipient (vehicle-treated) mature animals did not change lesion growth, whereas deletion of ER β in recipient animals reduced lesion size slightly (Fig. 8A). In α KO recipients, OBHS lost almost all inhibitory effects observed in wild-type recipient mice, whereas knockout of ER β resulted in only a slight loss in OBHS effectiveness, suggesting that ER α is the predominant ER mediating the effects of OBHS, consistent with its fivefold higher affinity for ER α (13). In contrast, CLI, which binds very preferentially (about 70 $\times$) to ER β (10), appeared to be acting predominantly through ER β , with greater loss of effectiveness in β KO than in α KO animals (Fig. 8A).

The effects of CLI or OBHS on inflammatory responses in KO mice were mirrored by congruent changes in cytokine production in lesions. Because systemic blood levels of E₂ are very high in α KO females owing to loss of the central feedback regulation normally exerted by ER α in the hypothalamus and pituitary, and consistent with a previous report (30), transcript levels of cytokines in lesions from α KO recipients were highly (about 14 $\times$)

elevated (Fig. 8B), whereas deletion of ER β in recipient animals had no effect on basal (vehicle) cytokine expression in lesions (Fig. 8C). In α KO recipients, a more obvious loss of suppressive effect by OBHS versus CLI in cytokine production was observed (Fig. 8B). In β KO mice, the suppressive effect of CLI on *Ccl2*, *Ccl5*, and *Il6* expression was completely lost, whereas OBHS only partially affected *Il6* expression (Fig. 8C). As expected, uteri of α KO mice were very small compared to those of wild-type or β KO recipient mice, which were similar in weight (fig. S13). In contrast to the suppressive effects of OBHS and CLI in lesions, neither ligand affected the weight of the uterus in wild-type, α KO, or β KO host animals (fig. S13).

DISCUSSION

Our findings, schematized in Fig. 8D, reveal that the two ER ligands, OBHS and CLI, are effective in suppressing murine and human endometriotic cell growth by coordinately affecting key aspects of disease progression: reducing proliferation, increasing apoptosis, and reducing estrogen-dependent inflammatory activities, vascularization, and innervation of lesions. These ligands display potent antiestrogenic and anti-inflammatory activities mediated via ERs in endometriotic cells and infiltrating macrophages. Many factors—genetic, environmental, immune, and endocrine—have been investigated for their role in the pathogenesis of endometriosis, yet the etiology of this disease and optimal approaches for its treatment still remain largely uncertain (2, 3). Our work suggests that modulation of ER signaling can provide a point of therapeutic intervention for many of the critical components of disease progression by using ligands that preferentially target the ER-inflammatory axis.

Medical treatments for endometriosis currently include three Food and Drug Administration–approved regimens: danazol (androgen), medroxyprogesterone acetate (progestin), and leuprolide (GnRH agonist). All three drugs inhibit pituitary gonadotropin secretion, preventing ovarian follicle growth and estrogen production. Aromatase inhibitors are also used off-label. Unfortunately, each of these agents occasions serious side effects including hirsutism, weight gain, or bone loss, limiting long-term use. Oral contraceptives and synthetic progestins have some efficacy, but half of symptomatic women fail to respond, presumably due to “progesterone resistance” (31).

We have used our knowledge of ER biology to develop new classes of therapeutics for endometriosis. We find that OBHS works more through ER α than ER β , whereas CLI works almost exclusively through ER β , consistent with their ER subtype binding selectivities (10, 13). In contrast to current therapeutics that suppress systemic estrogen levels, these two ER ligands display combinations of antiestrogenic and anti-inflammatory activities and appear to interrupt the extensive crosstalk among cell types comprising endometriotic lesions: donor uterine cells and infiltrating host cells from the immune, nervous, and vascular systems contributing to the growth of ectopic endometriotic lesions. Indeed, recent network analysis of clinical endometriosis indicates that inflammatory gene pathways support disease progression (32).

We found that proliferation, cytokine and chemokine production, NF κ B activation, immune cell infiltration, and COX2 expression were all suppressed by treatment with OBHS and

CLI, thus supporting the anti-inflammatory activities of these ligands. Considering the evidence that estrogen action not only is essential for endometriotic tissue growth but also contributes to ongoing inflammation, neovascularization, and associated pain (1, 8), our work in α KO and β KO mice indicates that OBHS and CLI intercept these intertwined inflammatory responses mainly by modulating ER activities.

We examined the effects of OBHS and CLI on macrophage involvement because we observed marked infiltration of macrophages into ectopic lesions, as found also in lesions and peritoneal fluid of endometriosis patients (33, 34). Our findings support the importance of macrophages in promoting lesion establishment and growth. Hence, optimal lesion growth with E_2 was facilitated by mature macrophages, which contain ERs and respond to E_2 (35), with macrophage-depletion studies revealing that these innate immune cells were major contributors to E_2 -stimulated cytokine production. Other immune cells (neutrophils, T and B cells, others) might also play roles in enhancing endometriosis. Indeed, T cells, which we found present in the endometriotic lesions, are reported to contain ERs (36, 37), and estrogen stimulation of endometriotic tissue promotes infiltration of various immune cells by enhanced production of chemotactic factors such as monocyte chemotactic protein-1 (38).

Compared with normal eutopic endometrium, the ectopic lesion, which appears particularly responsive to the actions of OBHS and CLI, has unusual steroid hormone production and receptor expression patterns, which contribute to abnormal hormone signaling in patients (8). Studies from murine models using ER knockout transgenic mice (30) and treatment with ER subtype-selective ligands (15, 39) further indicate crucial roles for ERs in endometriosis. Our work in α KO and β KO and EGFP host mice highlights that the endometriotic lesion provides a multicellular environment that has multidirectional interrelationships among the different cell types within the endometriotic lesion and the host background. In this regard, the failure of CLI to suppress lesion growth when ER β was deleted in the recipient mice highlights the essential role of ER β in mediating the actions of this very ER β -selective ligand (10). The ER β -dependent outcomes for CLI in our endometriosis model are consistent with the ER β -dependent effects of CLI in microglia cells and in a mouse model of multiple sclerosis (12). By contrast, ER α was the predominant mediator of OBHS effectiveness. We were not able to study the impact of our ligands on uterine donor tissue lacking ER α because, as shown by Burns *et al.* (30) previously, it is not possible to study α KO donor tissue in the endometriosis mouse model because the tissue is too atrophic to develop endometriotic lesions in host animals.

Development of a new vascular supply is required for endometriotic lesion establishment and survival (1). Using EGFP recipient mice, we found that substantial numbers of endothelial cells, together with immune cells and neurons, were recruited from the host into ectopic tissue. OBHS and CLI blocked production of angiogenic factors CYR61 and VEGFA by ectopic cells during initial lesion attachment, leading to reduced vascularity and severely impeded lesion establishment. Complete surgical eradication of endometriotic lesions is technically challenging and rarely achievable clinically. Given that disease symptoms commonly recur postoperatively or after cessation of treatment (1), our results suggest that OBHS and CLI offer promising therapeutic leads for long-term suppression of preexisting lesions and prevention of recurrence.

A major goal in medical treatment of endometriosis is reduction of symptoms, especially pain, which is partially responsive to therapies suppressing estrogen production (1). Although the hormonal mechanisms involved are not fully understood, observations in the clinic and in a rat model suggest that nerve fiber density innervating endometriotic tissue is correlated with pain (5, 21). In our model, newly established blood vessels, nerve fibers, and infiltrating immune cells were colocalized in ectopic tissue. Upon ligand treatment, decreased innervation and expression of NGF were coincident with suppressed vascularization and inflammation in regressed lesions, supporting the neuroangiogenesis hypothesis and suggesting future therapeutic potential of OBHS and CLI in relieving pain-associated symptoms in endometriosis. Because greater lesion regression and reduction of inflammation and innervation were observed when our ligands were given with the aromatase inhibitor letrozole, cotreatment with OBHS or CLI and an aromatase inhibitor might optimize clinical endometriosis management.

Because endometriosis primarily affects reproductive-age women, new therapeutics with preferential specificity for ectopic tissue and minimal effects on normal eutopic endometrium are desired, such that reproductive cycles and fertility remain unperturbed (1). OBHS and CLI treatment did not have deleterious effects on the reproductive tract or disturb estrous cycling or fertility, offering a clear advantage over current disease management with GnRH agonists or oral contraceptives. By their effectiveness in suppressing both estrogenic and inflammatory pathways, OBHS and CLI might prove to have excellent clinical efficacy in reducing disease burden, preventing disease recurrence, and reducing distressing endometriosis-associated symptoms such as pain. Future preclinical studies in nonhuman primates and safety trials will be needed to further determine the utility of these two compounds in treating clinical endometriosis in women. Because of their activity in suppressing estrogen-driven proliferation as well as inflammation, these compounds might also prove useful in the treatment of other disorders, such as multiple sclerosis (12), cardiovascular and metabolic effects related to obesity, certain cancers (such as inflammatory breast cancer), lung disorders such as lymphangioleiomyomatosis, and liver fibrosis, in which estrogens and inflammatory factors play key roles.

MATERIALS AND METHODS

Study design

Research objectives—Our studies were designed to evaluate the effectiveness of two ER ligands in preventing endometriosis lesion establishment and in eliciting lesion regression in a mouse model, and to examine the mechanisms underlying their antiproliferative and anti-inflammatory activities.

Experimental animals—Wild-type, EGFP-expressing, and α KO and β KO mice were used. All experiments involving animals were conducted in accordance with National Institutes of Health (NIH) standards for the use and care of animals, with protocols approved by the University of Illinois at Urbana-Champaign. C57BL/6 mice were purchased from Harlan Laboratories (wild type) or The Jackson Laboratory (Tg-ACTB-EGFP, stock no.

006567). α KO mice, β KO mice, and wild-type littermates were as described previously (40, 41).

Experimental design—Ectopic lesions were surgically introduced (14, 15). Uterine horns were removed from wild-type donor mice at diestrus (low estrogen stage), opened longitudinally, cut into fragments using a 3-mm dermal biopsy punch (Miltex), and transplanted onto the peritoneal wall of recipient mice by suturing. Sham control mice were subjected to the surgery, but in place of suturing uterine tissue, peritoneal tissue from a syngeneic donor mouse was used.

In each experimental group, uterine tissue was collected from at least six donors and transplanted into six recipients. After ligand treatments, host eutopic uterine tissue and ectopic tissues were collected. Ectopic lesion volume was calculated as a half ellipsoid that best approximated lesion shape on the peritoneum, using $V = (1/2)(4/3)\pi r_1^2 r_2$ (r_1 and r_2 are radii, $r_1 < r_2$). Sample size was set at six animals per group to detect typical differences in murine endometriotic-like lesion size and eutopic uterine weight (4, 42), accepting a conventional type I error of 5% and a type II error of 10%.

In the prevention model, ligands were administered to ovariectomized recipient mice by subcutaneous implantation of compound-releasing 20-mg cholesterol pellets (Sigma-Aldrich). For 2-week studies, E_2 (Sigma-Aldrich) dosage (0.125 mg per pellet) was chosen on the basis of other reports (43) and our findings. OBHS or CLI (0.25 mg per pellet) was coadministered with E_2 to test their antiestrogenic effects. In the therapeutic model, 0.25 mg of OBHS or CLI per pellet per animal was used on the basis of dose-response studies in Fig. 2B. For long-term treatment (up to 6 weeks), pellets were replaced every 2 weeks.

In studies to deplete macrophages from mice, clodronate-containing liposomes (ClodronateLiposomes.org) were used. Each mouse received five 200- μ l intraperitoneal injections of clodronate-containing liposome preparation (5 mg of clodronate-liposomes per milliliter of phosphate-buffered saline) over a 13-day period (as shown in Fig. 7A).

Primary hESC cultures and treatments

Our studies involving human endometriotic lesion biopsies and primary cell cultures were approved by the Institutional Review Boards of the University of Illinois and Wake Forest University School of Medicine. All protocols adhere to the regulations set forth for the protection of human subjects participating in clinical research, including the establishment of a data and safety monitoring plan. Human endometriosis stromal cell cultures were prepared from biopsies of endometrioma cyst linings and cultured as described (44).

Cell viability assays

WST-1 assay (Roche) was performed in triplicate to quantify cell viability. Absorbance was read at 450 nm on a Victor X5 plate reader (PerkinElmer). Assays were independently conducted at least three times.

Apoptosis assays

TUNEL staining was used to assess apoptosis activity in cultured cells and ectopic tissue sections with the In Situ Cell Death Detection Kit (Roche) (45).

Histological analyses

Immunofluorescence, immunohistochemistry, H&E staining, and whole mount staining were performed in cultured cells or paraffin-embedded mouse tissue sections (45). Primary antibodies are listed in table S2. Stain signal was quantified by monitoring the average numbers of positively stained cells relative to the total number of cells from six randomly chosen fields.

RNA isolation and real-time RT-PCR

Total RNA was isolated using TRIzol reagent (Life Technologies) to prepare complementary DNA. Real-time PCR was performed to quantify gene expression using specific primers (table S3) and SYBR Green (Bio-Rad). After analysis by the C_T method, data were normalized to 36B4 expression (45).

Liquid chromatography–mass spectrometry analysis of CLI and OBHS

Serum samples were analyzed for CLI and OBHS with the 5500 QTrap liquid chromatography–mass spectrometry system (AB Sciex) and a 1200 series high-performance liquid chromatography system (Agilent), with separation on a Phenomenex C6-Phenyl column.

Statistical analysis

Statistical analysis used one-way ANOVA with Bonferroni's multiple comparison test or two-way ANOVA with Bonferroni posttest in GraphPad Prism 5.00 (GraphPad). In studies with α KO and β KO and wild-type littermates, one-way ANOVA was performed to address the influence of genotype. The effect of OBHS or CLI was considered in each genotype using Bonferroni posttest. Data are expressed as means $\pm$ SD, with $P < 0.05$ considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Effects of OBHS and CLI in the endometriosis prevention model

(A) Uterine fragments from intact donor mice were engrafted to the peritoneal wall of ovariectomized syngeneic recipient mice, which were then treated with ligands or control vehicle (Veh; $n = 6$ mice per group) for up to 14 days. (B) Structures of compounds. (C) Ovariectomized recipient mice were treated with control vehicle, E_2 (0.125 mg per pellet), $E_2 + OBHS$, or $E_2 + CLI$ (0.125 mg of E_2 and 0.25 mg of OBHS or CLI per pellet), and ectopic lesion growth was measured over the 14 days of treatment. * $P < 0.05$ [$n = 6$ per group, two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test].

Original data for each animal are given in table S4, and exact *P* values in table S5. **(D)** H&E staining of ectopic tissues from recipients treated with different ligands or control vehicle for 14 days. Scale bar, 200 μ m. **(E)** Immunohistochemistry of Ki67 in ectopic tissue after 14 days of treatment. Scale bar, 100 μ m. **(F)** Quantification of Ki67-positive cells as a percent of the number of total cells in lesions ($n = 6$ per group). **(G)** PECAM immunofluorescence was analyzed to observe the vasculature in ectopic lesions after 14 days of ligand treatment ($n = 6$ per group). Scale bar, 100 μ m. DAPI, diamidino-2-phenylindole. **(H)** Quantification of microvessel density in ectopic lesions at day 14 ($n = 6$ per group). **(I)** Level of *Cyr61* and *Vegfa* profiled by quantitative polymerase chain reaction (PCR) ($n = 6$ per group). **(J)** Immunohistochemistry for CYR61 and VEGFA protein in ectopic tissues after 3 days of vehicle or ligand treatment ($n = 6$ per group). Scale bars, 100 μ m. BV, blood vessel; C, cyst; E, epithelial cells; S, stromal cells; P, peritoneal wall. Lowercase letters indicate $P < 0.05$ by one-way ANOVA with Bonferroni's multiple comparison test.

Fig. 2. Effects of OBHS and CLI in the therapeutic model

(A) Uterine donor tissue was transplanted to the peritoneal wall of ovary-intact recipient (8-week-old) female mice. After 2 weeks of lesion establishment, recipient animals received OBHS or CLI or control vehicle for up to 6 weeks. Both ectopic and eutopic tissues were collected from recipients at diestrus. (B) Effect of ligand dosage (0, 0.1, and 0.25 mg per pellet) on lesion growth and eutopic recipient uterine weight at 6 weeks of ligand or vehicle treatment ($n = 6$ per group). Lowercase letters indicate $P < 0.05$ by two-way ANOVA with Bonferroni's multiple comparison test. (C) Lesion volume was monitored over the 6 weeks of ligand treatment (0.25 mg per pellet, $n = 6$ per group). $*P < 0.05$. Original data for each animal are given in table S4, and exact P values in table S5. (D) Immunohistochemistry of Ki67 in ectopic tissue after 6 weeks of treatment. Scale bar, 50 μm. (E) Quantification of Ki67-positive cells as a percentage of the number of total cells in lesions ($n = 6$ per group). (F) PECAM immunofluorescence to observe the vasculature in ectopic lesions after 6 weeks of treatment. Scale bar, 50 μm. (G) Quantification of microvessel density in ectopic lesions

at 6 weeks ($n = 6$ per group). $*P < 0.05$ versus vehicle. Lowercase letters indicate $P < 0.05$ by one-way ANOVA with Bonferroni's multiple comparison test.

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Fig. 3. Anti-inflammatory effects of OBHS and CLI

(A) Expression of cytokines in ectopic tissues was analyzed by quantitative reverse transcription PCR (RT-PCR) over 6 weeks of treatment. mRNA levels are expressed relative to transcript level in eutopic uterine donor tissue, set at 1.0 ($n = 6$ per group). * $P < 0.05$ by two-way ANOVA with Bonferroni's multiple comparison test. Exact P values are given in table S5. (B) IL-6 immunohistochemistry in ectopic lesions after 2 weeks of treatment. immunoglobulin G (IgG) served as negative control for the anti-IL-6 antibody. Scale bar, 50 μm. (C) Quantification of IL-6 staining signal in ectopic lesions ($n = 6$ per group). (D)

NF κ B activity monitored by immunostaining of p65 in ectopic lesions after 2 weeks. Scale bar, 50 μ m. (E) Quantification of p65 nuclear staining in ectopic lesions ($n = 6$ per group). (F) COX2 immunofluorescence in ectopic lesions after 2 weeks of treatment. Scale bar, 200 μ m. (G) Fluorescence images of TUNEL staining in ectopic lesions. Scale bar, 50 μ m. (H) CD3 and F4/80 immunofluorescence to monitor T cells and macrophages, respectively, in ectopic lesions, and quantification of CD3- and F4/80-positive cells ($n = 6$ per group). Scale bar, 50 μ m. E, epithelial compartment; S, stromal compartment. Lowercase letters indicate $P < 0.05$ by one-way ANOVA with Bonferroni's multiple comparison test.

Fig. 4. Effects of ligands on innervation and neuron activation in ectopic endometriotic lesions (A and B) Dual immunofluorescence of PGP 9.5 and SP (A), or PGP 9.5 and CGRP (B), in ectopic lesions after 2 weeks of treatment with vehicle or ligand. Costaining signals are highlighted by white arrows. (C) Double immunofluorescence of NGF and NGFR-p75 in ectopic lesions. Costaining signals are highlighted by white arrows. Scale bars, 50 μm.

Fig. 5. Cotreatment with letrozole in combination with ligands in the therapeutic model (A and B) Ectopic lesion volume (A) and eutopic uterine weight (B) after 2 weeks of letrozole (0.75 mg per pellet) with or without OBHS or CLI ligand (0.25 mg per pellet), or with OBHS or CLI alone ($n = 6$ per group). Original data for all animals are given in table S4. (C) Proliferation monitored by Ki67 staining ($n = 6$ per group). (D) Quantitative RT-PCR analysis of cytokine mRNA expression in ectopic tissues, with transcript level in vehicle-treated tissue set at 1.0 ($n = 6$ per group). (E) Angiogenesis and innervation were assessed by immunofluorescence of PECAM and PGP 9.5 after 2 weeks. Scale bars, 50 μ m.

Let, letrozole. Lowercase letters indicate $P < 0.05$ by one-way ANOVA with Bonferroni's multiple comparison test. Data for Ki67 staining, cytokine expression, angiogenesis, and innervation for lesions after OBHS or CLI treatment alone were shown in Figs. 2 to 4.

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Fig. 6. Effects of OBHS and CLI on hESCs in vitro

(A) Primary cultured hESCs were treated with ligands for 6 days, and cell proliferation was monitored ($n = 6$ per group). (B) Immunofluorescence of phosphorylated Ser¹⁰ of phospho-histone 3 (p-H3) was performed to monitor the mitotic activity of endometriotic stromal cells after 24 hours. p-H3-positive nuclei are highlighted by white arrows ($n = 6$ per group). Scale bar, 50 μ m. (C) Quantification of p-H3-positive cells ($n = 6$ per group). (D) Apoptotic DAPI-stained nuclei are red by TUNEL assay after 72 hours. White arrows indicate apoptotic foci. Scale bar, 50 μ m. (E) Percentage of apoptotic cells ($n = 6$ per group). (F)

Cytokine mRNA levels in hESCs after treatment for 24 hours ($n = 6$ per group). E₂, 10 nM; TNF α , 20 ng/ml; OBHS, 1 μ M; CLI, 1 μ M; ICI, 1 μ M. Lowercase letters indicate $P < 0.05$ by one-way ANOVA with Bonferroni's multiple comparison test.

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Fig. 7. Role of macrophages in the prevention of lesion establishment by OBHS or CLI
(A) Serial injections of clodronate-containing liposomes (Clod; 1 mg per injection) were used to deplete macrophages from ovariectomized recipient mice. Donor tissues were transplanted followed by 2 weeks of OBHS or CLI treatment. **(B)** Lack of F4/80 staining, quantified in stroma, confirmed the absence of macrophages in ectopic tissue in Clod-treated hosts. Scale bar, 50 μ m. S, stromal compartment. **(C)** Lesion volume with and without clodronate and treatment with vehicle, E₂, E₂ + OBHS, or E₂ + CLI for 2 weeks ($n = 6$ per group). **(D)** Quantitative RT-PCR analysis of cytokine transcript levels in ectopic lesions,

with transcript level in vehicle-treated lesions without clodronate set at 1.0 ($n = 6$ per group). (E) Eutopic uterine weight with and without clodronate and treatment with vehicle, E_2 , $E_2 + OBHS$, or $E_2 + CLI$ for 2 weeks ($n = 6$ per group). Original data for all animals are given in table S4. Lowercase letters indicate $P < 0.05$ by one-way ANOVA with Bonferroni's multiple comparison test.

Fig. 8. Effectiveness of ligand treatment against lesion progression in ER transgenic mice
 Intact wild-type (WT), αKO, or βKO mice were used as recipient animals ($n = 6$ per group). (A) Lesion volume was quantified after 2 weeks of lesion establishment from WT donor uterine tissue and 2 weeks of vehicle, OBHS, or CLI treatment (0.25 mg per pellet). P values determined by one-way ANOVA with Bonferroni's multiple comparison test. (B and C) Quantitative RT-PCR analysis of relative mRNA expression levels of cytokines in ectopic tissue. Transcript level in vehicle-treated WT ectopic tissue transplanted in WT recipients was set at 1.0 ($n = 6$ per group). Note different y-axis scales in (B) and (C). (D)

Schematic model depicting the mechanisms by which OBHS and CLI exert their antiestrogenic and anti-inflammatory effects via ERs to block events contributing to establishment and progression of endometriosis.

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