

REVIEW ARTICLE

Brain-Targeted Delivery of Estradiol: Therapeutic Potential and Results Obtained with a Chemical Delivery System Approach

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Abstract

Estradiol is the most potent human estrogen, and because many of its pharmacological effects are mediated through the central nervous system (CNS), there are several potential therapeutic applications for a system that can achieve the brain-targeted delivery. By directing the active compound away from the circulatory system and behind the blood-brain barrier, brain-targeting should increase treatment efficiency for the CNS-mediated effects and reduce the likelihood of peripheral side effects, such as an increased risks for cancer (the main reason many women avoid traditional hormone therapy). Possible therapeutic application include the treatment of menopausal vasomotor symptoms (hot flashes), the treatment and/or prevention of various types of dementia (including Alzheimer disease), treatment of male or female sexual dysfunction, and possibly neuroprotection. Preclinical and clinical results obtained with an estradiol chemical delivery system, an innovative chemical entity that uses a redox CDS approach to selectively target estradiol to the brain and the CNS by releasing it following a designed metabolic sequence, demonstrate effective brain-targeting and long-lasting pharmacological activity, thus confirming the potential of this approach.

Estrogens are endogenous hormones that produce numerous physiological actions and are among the most commonly prescribed drugs in the US, mainly for hormone therapy (HT) in

postmenopausal women and as a component of oral contraceptives.^[1] Menopause-related estrogen depletion is considered to be associated with a variety of symptoms that

range from vasomotor complaints to cognitive effects. Traditional HT may alleviate many of these complications, but is avoided by many women because of the increased risks for cancer, stroke, and other diseases.^[2-6] Furthermore, HERS (Heart and Estrogen/Progestin Replacement Study) suggested that, contrary to earlier expectations, HT did not in fact decrease the incidence of coronary heart disease (CHD).^[7,8]

Two large-scale, parallel, randomized, double-blind, placebo-controlled clinical trials were undertaken within the Women's Health Initiative to determine whether conjugated estrogens (i.e. obtained from pregnant mares and commonly used in HT) alone in women with prior hysterectomy or in combination with the progestogen medroxyprogesterone reduce cardiovascular events in postmenopausal women; both studies were halted early because of unfavorable outcomes.^[9-11] The estrogen plus progestogen component, which had a planned duration of 8.2 years and had recruited 16 608 patients, was stopped prematurely because it indicated an unacceptably increased risk (26%) of invasive breast cancer.^[9,10] The estrogen plus progestogen component was also associated with a 41% increased risk of stroke and a 29% increased risk of developing CHD. The estrogen alone component, which enrolled 10 739 women, was also ended early as it increased the risk of stroke (hazard ratio of 1.39) and did not affect the incidence of CHD (although it did decrease the risk of hip fracture).^[11]

Data from these studies are undisputable and statistically well supported; nevertheless, some of the main conclusions are still controversial – a major criticism being that these studies included women much older (average of 64 years of age) than the typical HT user, who tends to initiate therapy around the time of menopause (average of 51 years of age). There is some evidence of a 'window of opportunity,' in that women who initiate HT later could miss the chance to really benefit from the treatment. Consequently, studies and trials focusing on the timing of HT have already been initiated.^[12,13] It is also very much possible that the adverse effects of xenoestrogens and progestogens revealed by these studies do not necessarily generalize to the use of human formulations. For example, a population-based, case-control study found that conjugated estrogens, but not esterified estrogen, were associated with increased venous thrombotic risk (odd ratios of 1.65 vs. 0.92 compared with women not using hormones).^[14]

In light of these results and the approximately 30% increase in the risk of breast cancer caused by taking estrogens, which may rise to 50% if taken for more than 10 years,^[15] there is currently little justification for systemic HT. Not surprisingly, a nationally representative US survey^[16] of women >50 years old found that hormone use dropped to 12% in the first half of 2004 from 28% in 2002, and according to IMS Health, the number of US prescriptions dispensed (estrogens) dropped to 24.7 million in the first eight months of 2005 from 45.2 million in the same period in 2002.^[16]

Whether or not to take HT is an important decision because in industrialized nations the average woman spends around a third of her life in the postmenopausal stage.^[17] As the central nervous system (CNS) is the target site for many estrogenic actions,^[18-21] brain-targeted delivery may provide safer and more effective agents in many cases. By directing the active compound away from the circulatory system and behind the blood-brain barrier (BBB), brain-targeting should reduce circulating hormone levels, and, thus, the incidence of cancer and most likely even stroke. Additionally, with the recent unraveling of the role of estrogens in males,^[22,23] it is clear that there could be a number of possible therapeutic applications in males as well.

1. Brain-Targeted Delivery of Estradiol: Therapeutic Potential

Considering the distribution of estrogen receptors (ER) in the brain, a variety of potential therapeutic applications can be envisaged for brain-targeted estrogens. ERs are found in the hypothalamus and the closely associated preoptic area, where they mediate the effects of estradiol on luteinizing hormone (LH) secretion, sexual behavior (both in males and females), and appetite and temperature regulation.^[18,24] ERs have also been identified on dopaminergic neurons that innervate the striatum where estradiol may modulate locomotion, and, hence, may be involved in movement disorders.^[19,25] They have also been identified in mesocortical dopaminergic neurons, where they may mediate the effects of estradiol on mood, and in the region of the nucleus basalis magnocellularis (a site of cholinergic cell bodies that enervate the cerebral cortex), where they may mediate the influence of estrogens on cognitive functions.^[25] Estrogens promote neuronal branching and density, particularly in the CA1 area of the hippocampus and the basal frontal cortex.^[21,26-29] Estrogen also interacts with several neurotransmitter systems, such as those using acetylcholine, norepinephrine, serotonin, and others. Consequently, there is an increasing tendency to expand the usually restrictive view of estrogen action in the brain beyond the confines of sexual differentiation and reproductive neuroendocrine function, and to consider the much broader question of estrogens as neural growth factors with important influences on the development, survival, plasticity, regeneration, and aging of the mammalian brain.^[30-32]

ERs may not only be ligand-induced transcriptional enhancers but also a mediator of rapid, nongenomic events, such as the excitability of neuronal and pituitary cells and the modulation of G-protein coupling.^[32] Furthermore, the antioxidant and neuroprotective effects of estradiol may also involve other receptors and may be independent from estrogenic properties.^[33-37]

Consequently, brain-targeted estrogens could be useful in reducing the secretion of LH-releasing hormone (LHRH) and, hence, in reducing the secretion of LH and gonadal steroids. As

such, they could be employed to achieve contraception and to reduce the growth of peripheral steroid-dependent tumors, such as those of the breast, uterus, and prostate, and to treat endometriosis. They also could be useful in stimulating male and female sexual behavior, and in the treatment of menopausal vasomotor symptoms (e.g. hot flashes).^[38] Other potential uses are for neuroprotection, reduction of body weight, treatment of depression and schizophrenia,^[19] and various types of dementia, including Alzheimer disease.^[4,39-46]

1.1. Menopausal Syndrome

The menopausal syndrome defines a constellation of syndromes associated with the decline of ovarian function.^[17] The most common symptoms are hot flashes, perspiration, muscle and joint pain, headaches, and irritability. Their intensity and frequency vary, and symptoms do not occur in about 25% of menopausal women in western countries,^[17] the average age at menopause is about 51 years. The most dramatic hormonal alteration at the menopause is an almost 20-fold decline in the circulating levels of estradiol. Premenopausal estradiol levels are cycle-dependent and fluctuate between 20 and 400 pg/mL; following menopause, these decrease to 5–25 pg/mL.^[11] Interestingly, in men, estradiol is maintained at a much more constant level (10–60 pg/mL) as it is mainly produced by aromatizing testosterone, and while testosterone production gradually declines with age, it never ceases.^[47,48] Compared with age-matched women not using estrogen, healthy elderly men have significantly higher plasma estradiol levels, and this might have some protective effects, for example, on bone density or explicit memory.^[48]

The abrupt decline of estrogen levels in women appears to be the most important factor in the genesis of hot flashes, and estradiol (administered as valerate, benzoate, or enanthate formulations) is effective in alleviating hot flashes.^[49] Oral HT is highly effective in alleviating hot flashes and night sweats.^[50] Transdermal estradiol patches are also effective in reducing hot flashes in menopausal women^[51,52] and in men undergoing hormonal therapy for prostate cancer.^[53] The usual effective dose for reducing menopausal symptoms is considered to be 25–50 µg/day (transdermal); higher dosages using an estradiol transdermal system (50–100 µg/day) were accompanied by estrogen-related adverse effects.^[51] The brain-targeted delivery of estradiol could provide brain drug concentrations that are sufficient to ameliorate the symptoms of hot flashes, while also avoiding peripheral estrogen toxicity. Trickle-down concentrations, resulting from the slow release of the active hormone from the brain to the peripheral circulation, may be sufficient to avoid osteoporosis.

The main risk factors for hip and related fractures are low bone density and falls, both of which become increasingly common with advancing age. HT significantly decreases the

hazard ratio for hip fractures.^[9] However, part of the fracture-protecting effect of estrogens might result not just from the effect on bone density but also from a CNS-mediated effect on postural balance,^[20,54] as the incidence of falls among women after menopause is higher than in men. Such a fracture-preventing effect has shown in some studies,^[54] but not confirmed by others.^[55,56]

1.2. Male and Female Sexual Dysfunction

Following the introduction of sildenafil for the treatment of erectile dysfunction in 1998, there has been increasing focus on the pharmaceutical treatment of male and female sexual dysfunction. Female sexual dysfunction (FSD), including sexual pain and disorders of desire, arousal, and orgasm^[57] represent an obvious potential therapeutic area for estradiol. Hypoestrogenism is the physiologic condition most commonly associated with FSD,^[57] and a strong influence of estradiol on female sexual behavior has been confirmed in many animal models.^[58,59] While there is currently no consensus, evidence suggests that decline in female sexual function with natural menopausal transition is more closely related to a decrease in estradiol levels rather than androgen levels.^[60] Therapeutic options for FSD (e.g. estrogen-androgen combination HT, tibolone, or sildenafil) show promise, but this is a complex problem and there are no clear answers or easy solutions.^[57,61]

In the US, more women (43%, about 40 million) than men (31%) experience some form of sexual disorder and, in contrast to men, the distribution of FSD is fairly even among women ranging from 18 to 59 years of age.^[62] Recently, we found that brain-targeted estradiol has a dramatic effect on the sexual behavior of female rats, which, considering the complexity of FSD, is a somewhat surprising, but quite possibly, significant finding (see section 3.3.2).^[63]

Intriguingly, various aspects of male sexual behavior also seem to depend on the local production of estrogens within the brain, a fact confirmed in many mammals.^[22] In addition to a few older studies investigating the role of estradiol benzoate in castrated male rats,^[64,65] a number of recent studies clearly highlighted the important sexual behavior-influencing role for estrogens in males.^[66] For example, male sexual behavior (mounting, intromission, and ejaculation) was completely disrupted in knock-out mice lacking both estrogen receptor α and β ($\alpha\beta$ ERKO),^[67] as well as in aromatase knock-out (ArKO) mice.^[66,68] In addition, estrogen therapy of a human male with aromatase deficiency (and, consequently, initially undetectable serum estradiol levels) resulted in increased libido, frequency of intercourse, masturbation, and erotic fantasies.^[69] Results from our research also confirmed the potential of brain-targeted estradiol to significantly influence male sexual behavior.^[63,70]

1.3. Alzheimer Disease

In a somewhat ironic twist, estrogens, often blamed for causing ‘raging hormonal imbalances’, are now being investigated for their possible cognitive functions.^[31,71] Estrogens, among others, seem to be essential substances for the maintenance of limbic brain function, thus regulating memory, emotion, orientation in time and space, motivation, and other cognitive functions in menopausal women. Thus, estrogen therapy may help delay processes of Alzheimer disease (AD) in postmenopausal women (Figure 1).

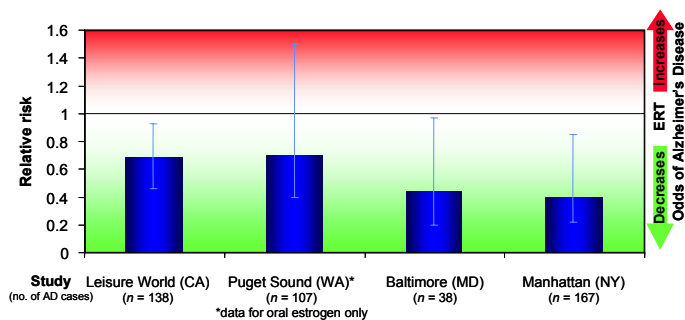


Figure 1. Relative risk estimates for Alzheimer disease (AD) associated with postmenopausal use of estrogen therapy. Relative risk indicates the odds of getting AD in the hormone therapy (HT) group versus the corresponding control group; therefore, a relative risk smaller than 1 indicates reduced odds of developing AD in the HT group. Data are from four large epidemiological studies, and error bars represent the 95% confidence intervals. * indicates data from oral estrogen only (reproduced from Henderson,^[28] with permission).

AD results in a progressive worsening of symptoms ranging from memory loss to declining cognitive ability; it affects an estimated 15 million people worldwide.^[72] AD is the leading cause of persistent dementia in later life,^[73,74] therefore, it is becoming a rapidly escalating burden with the ageing of the population in industrialized nations. Survival for a decade is common, and the prevalence of AD increases from ~3% at the age of 65 years to ~47% at the age of 85 years.^[72] Following cancer, AD is the second-most expensive disease in the US (more expensive than diabetes mellitus, for example) with an estimated yearly cost of around US\$100 billion, if both direct and indirect medical costs are included.^[75]

There are approximately twice as many women as men with AD and, although women on average live longer than men, most studies indicate the prevalence in women is higher, even after adjusting for differences in age distribution.^[28,76,77] During the course of the illness, women also tend to show greater impairments in naming tasks and other measures of semantic memory.^[28] The abrupt decline of estrogenic production in postmenopausal women is a suspected contributing factor.^[21,28,78]

The connection between sex hormone levels and cognitive function in men and women is a somewhat controversial area, but one with many intriguing aspects. A significant overlap

notwithstanding, gender differences in certain cognitive abilities (e.g. spatial tasks and verbal abilities) have been consistently found and are well documented; they arise at least partially because of differences in sex-steroid influences during both the early organizational perinatal period and then later in adult life.^[21,79,80] For example, on average, men perform better than women in certain spatial tasks such as those related to three-dimensional mental rotation and target-directed motor skills or in mathematical reasoning, whereas women, on average, perform better in verbal memory, mathematical calculation, and precision manual tasks (see Sherwin^[21] and Kimura^[79,80]). Testosterone levels, in addition to their well known connection to aggressive behavior,^[81] have been found to positively correlate with visuo-spatial ability and negatively correlate with verbal fluency, both in European men and in Namibian bushmen.^[82] Testosterone treatment also caused a clear, long-term improvement in spatial ability in female-to-male transsexuals,^[83,84] increased aggression and decreased verbal fluency were also seen in one trial,^[83] but not confirmed in a second.^[84] These relationships are probably not simple linear ones,^[85] most likely, there is some optimal androgen level (probably somewhere in the low range for males) because women with high levels and men with low levels tend to perform better on spatial tasks.^[79,80] Compared with men, on average, women are not superior on most verbal tests, but perform consistently better on verbal memory and verbal fluency tests.^[79,80]

There is also evidence that women’s performances at certain tasks change through the menstrual cycle as estrogen levels vary, with high estrogen levels around mid-cycle depressing spatial ability and enhancing speech and manual skill abilities.^[86-88] There is also fairly consistent evidence that estrogens maintain or improve verbal memory in postmenopausal women.^[89] This also appears to be the case in more special populations, for example, in male-to-female transsexuals,^[83,90] young girls with Turner’s syndrome,^[91] or women with uterine leiomyomata treated with leuporelin depot and showing symptoms of hypoestrogenism.^[92]

Estrogen is also more than likely involved in improving sense of smell. The superiority in women’s olfactory capacity compared to that of men’s arises during puberty, and sense of smell is keenest around ovulation, when estrogen levels are high.^[93] Memory and smell sensation are closely linked; there are even indications that AD originates with a decline in olfactory capacity.^[93] Abrupt changes in estrogen levels might also be implicated in the possible acceleration of age effects on cognition following menopause.^[78] A magnetic resonance imaging (MRI)–based study found that women taking HT had larger right hippocampal volumes than women not taking HT and larger anterior hippocampal volumes than men and women not taking HT.^[94] Because the hippocampus, a brain structure involved in memory, has been shown to shrink in patients with AD, these findings may also suggest that estrogen has a neuroprotective effect. Functional MRI studies have also confirmed that

estrogens affect brain activation patterns during working memory tasks.^[95]

Notwithstanding, estrogens produce many effects that may prevent or delay the onset of AD,^[18,21] or improve quality of life for patients. Brain areas known to support memory were found to have high densities of ER.^[18,24] Estrogens promote the growth and survival of cholinergic neurons, and also modulate serotonergic and catecholaminergic neurotransmission.^[18,24] Estrogens also increase cerebral blood flow and have antioxidant, anti-inflammatory, and general neuroprotective activity.^[18,36,96] A number of studies have found that estrogens decrease cerebral amyloid deposition and protect against β -amyloid-induced cell death not only in cell cultures,^[97,98] but also in animal models.^[99-101]

In guinea pigs, it was found that prolonged ovariectomy increased brain β -amyloid levels on average by 1.5 fold, and estradiol administration significantly reversed this increase.^[99] Furthermore, even human studies suggest that levels of circulating β -amyloid may be under the control of gonadal hormones.^[102] Because, an imbalance in β -amyloid production and clearance is suspected to result in a cascade of events ultimately leading to neuronal dysfunction and dementia (the amyloid cascade hypothesis of AD),^[103] estrogens are expected to have disease-modifying effects. In patients with AD, there is also a general trend toward lower levels of neurosteroids in different brain regions.^[104]

Actual findings on the effect of estrogens in AD are somewhat conflicting.^[105] There have been a number of small- and large-scale studies suggesting that estrogens have beneficial effects in patients with AD (Figure 1),^[4,39-46,106,107] in Parkinson's disease,^[108,109] and on mental performance in general.^[89,90] There is also evidence that women with AD receiving HT experience a better response to treatment with cholinesterase inhibitors than those not receiving HT.^[110] Until the 2003 approval of the *N*-methyl-D-aspartate (NMDA) antagonist memantine, cholinesterase inhibitors were the only US FDA-approved therapeutic drug class for patients with AD. This further confirms estrogenic interactions with cholinergic mechanisms, which are critically involved in attention processes, learning, and memory (domains particularly stricken by AD). In some studies, lower estradiol levels were also found to correlate with poor cognitive, behavioral, and functional status in older women^[111,112] and men.^[112]

On the other hand, the large-scale Alzheimer's Disease Cooperative Study^[76] found that HT with conjugated estrogens for 1 year did not slow disease progression or improve global, cognitive, or functional outcomes in women with mild-to-moderate AD. However, the study was conducted with oral estrogen dosages of 0.625 or 1.250 mg/day, which more recent studies^[45] suggest do not provide sufficiently elevated estradiol levels in the brain to affect cognitive processes; mean serum estradiol levels were 22.7, 48.0, and 58.4 pg/mL in the placebo,

low-dose estrogen, and high-dose estrogen treatment groups, respectively, i.e. at the low end of normal, premenopausal levels and close to what are usually considered post-menopausal levels.

High-dose estradiol (0.10 mg/day skin patches resulting in plasma levels >100 pg/mL) was found to improve cognition for women with AD in a randomized clinical study.^[113] According to one estimate, estradiol-levels of at least 20–40, 70, and 100 pg/mL are required for osteoporosis prevention, cardiovascular prevention, and cognitive enhancement, respectively.^[114]

In the large-scale Womens' Health Initiative Memory Study (WHIMS), conjugated estrogens alone^[115,116] or in combination with progestogen^[117,118] were not found to improve cognitive function or reduce dementia or mild cognitive impairment compared with placebo (in fact, slightly increased both). Low CNS levels and the late initiation of HT were the likely causes for these negative results.^[117] A pilot study in 428 women also found that early initiation of HT may be beneficial, for some cognitive domains, while the initiation of HT in late menopause may be detrimental.^[13] In addition, conjugated estrogens contain a combination of ≥ 10 estrogenic compounds (mainly estrone and equiline) in sulfated forms^[119,120] that, contrary to their nonsulfated forms, do not cross the BBB^[121] and are less likely to have CNS (e.g. cognitive) effects^[122] (eventually only after undergoing desulfation).

1.4. Neuroprotection

There is increasing evidence that estradiol has antioxidant and neuroprotective effects,^[123-125] neurotrophins may mediate some of the neuroprotective effects.^[125-127] *In vitro*, estrogens have been shown to have neuroprotective effects against the neurotoxicity that results from serum deprivation or β -amyloid and NMDA agonist treatment.^[97,98,128,129] A growing number of studies have demonstrated that exogenous estradiol reduces tissue damage resulting from experimental ischemic stroke in both sexes.^[129-132] ER α seems to have a critical role in mediating the protective effects of estradiol,^[133,134] however, the antioxidant and neuroprotective effects may also involve other receptors and may be independent from estrogenic properties.^[33-37]

2. Brain-Targeted Delivery of Estradiol: A Chemical Delivery System (CDS) Approach

Many pharmaceuticals cannot be effectively delivered and/or sustained within the brain and, therefore, are ineffective in the treatment of cerebral or CNS-related diseases. The main reason is the BBB, a unique membranous barrier that tightly segregates the brain from the circulating blood.^[135-137] Capillaries of the vertebrate brain and spinal cord are lined with a layer of special endothelial cells that lack fenestrations and are sealed with tight junctions. Therefore, only lipid soluble solutes that can freely

diffuse through the capillary endothelial membrane may passively cross the BBB, and lipophilicity (as measured by the log octanol-water partition coefficient, $\log P_{o/w}$) is a good predictor of BBB permeability.^[138,139] Not surprisingly, practically all drugs currently used for brain- or CNS-related disorders are lipid-soluble compounds that can readily cross the BBB following administration.

General strategies aimed at overcoming these problems and achieving brain-targeted delivery for other therapeutic agents as well have been reviewed previously.^[140]

2.1. Redox-Type CDS

Brain-targeted chemical delivery systems (CDS) represent a rational drug design approach that exploits sequential metabolism, not only to deliver but also to target drugs at their site of action.^[141-145] Localizing drugs at their desired site of action reduces toxicity and increases treatment efficiency. These brain-targeted systems exploit the specific properties of the BBB, not only to deliver therapeutic agents to the brain by increasing their lipophilicity, but also to 'lock' them within the brain and prevent them from re-crossing the BBB, thus providing sustained release. CDSs are inactive chemical derivatives of a drug obtained by one or more chemical modifications, so that the newly attached moieties are monomolecular units (generally comparable in size to the original molecule) and provide a site-specific or site-enhanced delivery of the drug through multistep enzymatic and/or chemical transformations. Most of these delivery systems have been reviewed in the literature.^[142-144]

Brain-targeting redox-type CDSs are obtained by chemically attaching a 1,4-dihydrotrigonelline (*N*-methyl dihydronicotinate) targetor (T) moiety to the original structure of a drug (D) and, if needed, some additional protective moieties. After administration, the resulting CDS is distributed throughout the body. Predictable enzymatic reactions convert the original CDS by removing some of the protective moieties and modifying the T moiety, leading to a precursor form (T^+-D), which is still inactive, but has significantly different physicochemical properties. While the charged T^+-D form is locked behind the BBB, it is easily eliminated from the body due to the acquired positive charge, which enhances water solubility. After a relatively short time, the delivered drug (as the inactive, locked-in T^+-D) is present essentially only in the brain, and carboxylic esterases-mediated hydrolysis of this intermediary T^+-D form provides sustained and brain-specific release of the active drug. The mechanism for estradiol-CDS is summarized in Figure 2. It has to be emphasized that the system not only achieves drug delivery to the brain, but provides preferential delivery, i.e. brain targeting. This should allow smaller doses and reduce peripheral adverse effects. Furthermore, since the 'lock-in' mechanism works against the concentration gradient, it provides more prolonged effects. Consequently, CDSs can be used not only to

deliver compounds that otherwise have no access to the brain, but also to retain lipophilic compounds such as estradiol within the brain.

2.2. Chemical Aspects

Estradiol is the most potent naturally occurring estrogen in humans. It contains two hydroxy moieties: one in the (phenolic) 3-position and one in the 17-position. With these synthetic handles, three possible CDSs can be designed attaching the targetor at either of the 17-, or 3-positions, or at both. Attachment at either position, but especially at the 17-position, should greatly decrease the pharmacological activity of estradiol, as these esters are known not to interact with ERs.^[146] A number of molecular manipulations have been explored for this CDS and the efficacy of 3-substituted versus 17-substituted,^[147] or the efficacy of different *N*-substituted targetor moieties (*N*-substituents range from methyl to hexyl or benzyl),^[148] have been evaluated. However, the, 17-(1,4-dihydrotrigonelline)-substituted estradiol-CDS remains the compound of choice.

Unfortunately, the same physicochemical characteristics that allow for successful chemical delivery also complicate the development of acceptable pharmaceutical formulations. The increased lipophilicity allows partition into deep brain compartments, but also confers poor aqueous solubility. The oxidative lability, which is needed for the 'lock-in' mechanism, and the hydrolytic instability, which releases the modifier functions or the active drug, combine to limit the shelf-life of the CDS. Cyclodextrins may provide a possible solution. Some of the early studies with estradiol-CDS were performed using a dimethyl sulfoxide vehicle; later, the corresponding inclusion complex with 2-hydroxypropyl- β -cyclodextrin (HP β CD) has essentially solved all the problems related to the formulation of estradiol-CDS.^[149] This modified cyclodextrin was selected based on its low toxicity observed using various administration routes and the fact that alkylation or hydroxyalkylation of the glucose oligomer can disrupt hydrogen bonding and provide increased water solubility for the cyclodextrin compound and its inclusion complexes.^[150-153] Indeed, the aqueous solubility of estradiol-CDS was enhanced about 250 000-fold in a 40% (w/v) HP β CD solution (from 65.8 ng/mL to 16.36 mg/mL). The phase solubility diagram indicated that a 1 : 1 complex forms at low HP β CD concentration, but a 1 : 2 complex occurs at higher HP β CD concentrations. The stability of estradiol-CDS was also significantly increased allowing formulation in acceptable form. The rate of ferricyanide-mediated oxidation, a good indicator of oxidative stability, was decreased about 10-fold, and shelf-life of estradiol-CDS was increased about 4-fold, as indicated by the t_{90} and t_{50} (the time required for 10% and 50% of the active principle to degrade) values in a temperature range of 23–80°C.^[149]

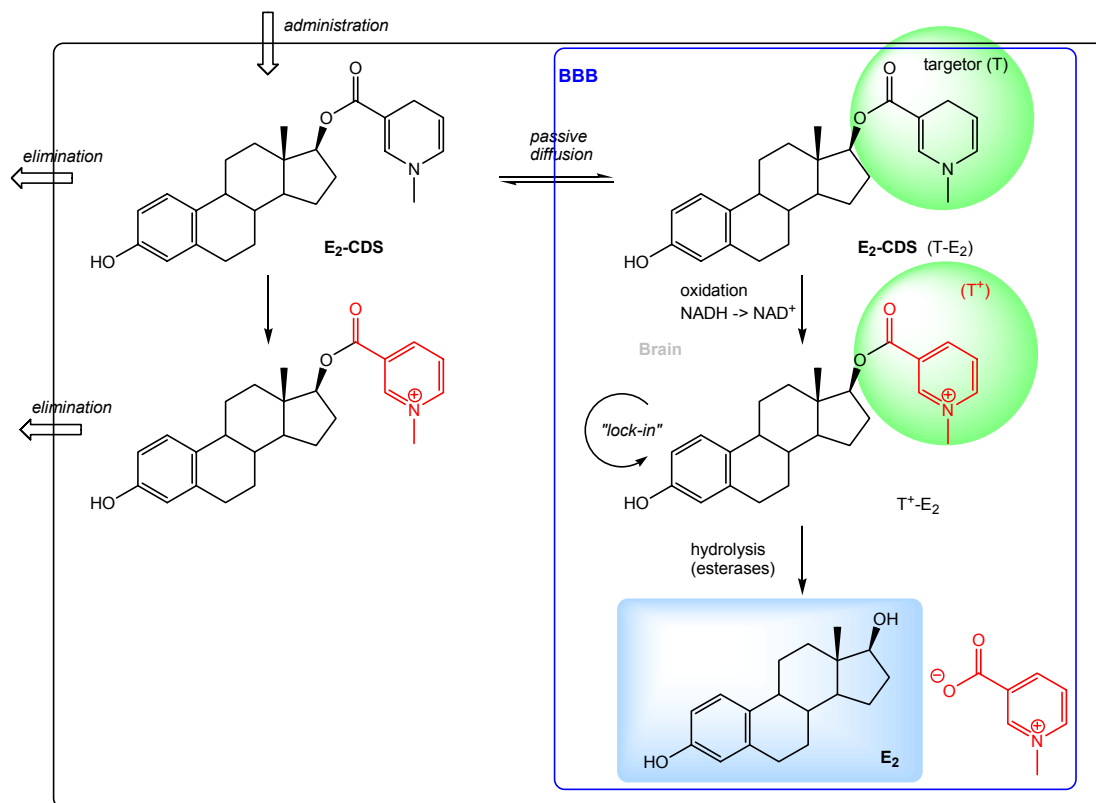


Figure 2. Illustration of the 'lock-in' mechanism for estradiol chemical delivery system (E₂-CDS). The lipophilic CDS (T-E₂, log *P* > 4) can easily cross the blood-brain barrier (BBB), but the hydrophilic intermediate (T⁺-E₂, log *D* < 0) is no longer able to come out providing a sustained release of the active estradiol (E₂). NAD = nicotinamide adenine dinucleotide.

3. Estradiol-CDS: Preclinical Investigations

3.1. *In Vitro* Studies

A CDS can accomplish its goal only if a relatively rapid T-D to T⁺-D conversion is followed by a slower T⁺-D to T⁺ + D conversion that releases the active drug from the 'locked-in' form in a sustained manner. *In vitro* studies using rat organ homogenates as the test matrix indicated half-lives of 156.6, 29.9, and 29.2 minutes for estradiol-CDS in plasma, liver, and brain, respectively.^[154] Thus, estradiol-CDS is converted to the corresponding quaternary form (T⁺-estradiol) faster in the tissue homogenates than in plasma. This is consistent with the hypothesis of a membrane-bound enzyme, such as a member of the nicotinamide adenine dinucleotide transhydrogenase family, acting as oxidative catalyst. These studies also indicated a very slow production of estradiol from T⁺-estradiol, suggesting a possible slow and sustained release of estradiol from brain deposits of T⁺-estradiol.

3.2. Tissue Distribution and Pharmacokinetics

To detect doses of estradiol-CDS (T-estradiol), T⁺-estradiol, and estradiol of physiological significance, a selective and sensitive method was needed. This was first accomplished using a precolumn-enriching high performance liquid chromatography (HPLC) system^[155] that allowed accurate detection in plasma samples and organ homogenates with limits of 10, 20, and 50 ng/mL (or ng/g) for T⁺-estradiol, estradiol-CDS, and estradiol, respectively. This study showed that in rats, estradiol released from the T⁺-estradiol intermediate, formed in the brain after intravenous estradiol-CDS administration, has an elimination half-life of more than 200 hours, considerably larger than the half-lives found in all the other tissues examined (e.g. kidney, heart, lung, testes, eye, and fat) that were all well below 50 hours, and brain estradiol-levels are elevated 4 to 5 times longer after estradiol-CDS administration than after simple estradiol treatment.^[155]

Proving effective targeting, another study also found that steroid levels between 1 and 16 days after estradiol-CDS treatment were more than 12-fold higher in brain samples than in plasma samples (see Figure 3).^[156] A consistent finding among

these studies^[49,155,157-159] is that the decline of T⁺-estradiol levels in the brain was considerably slower than in other tissues with relatively large initial concentrations (e.g. lung, kidney, and heart).^[158] This profile confirms that estradiol is indeed generated within the CNS and is not sequestered from peripheral sources.

Pharmacokinetically, serum estradiol data derived from estradiol-CDS administration could be fitted to a three exponent time-concentration curve. The initial phase ($t_{1/2} = 1$ h) was correlated with a rapid distribution phase, the second phase ($t_{1/2} = 9.9$ h) was associated with elimination of estradiol derived from several peripheral and central compartments, and the terminal elimination phase ($t_{1/2} = 123.5$ h) was associated primarily with elimination of estradiol derived from brain. In addition to rats,^[49,155,157,158] the pharmacokinetics of estradiol-CDS have also been examined in dogs.^[159] These data also confirmed the large volume of distribution for estradiol-CDS, its dose linearity, and slow terminal phase elimination.

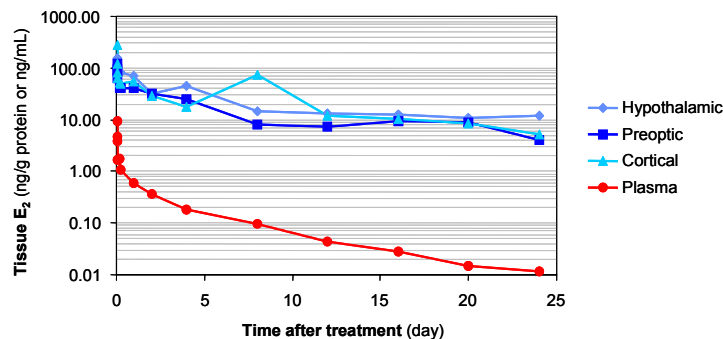


Figure 3. Concentration of estradiol in rat brain (cortex) and plasma^[156] after a single 3.0 mg/kg dose of estradiol-CDS.

3.3. Pharmacology

Studies in orchidectomized rats proved that a single intravenous (IV) injection of estradiol-CDS (3 mg/kg) suppressed LH secretion by 88%, 86%, and 66% relative to controls at 12, 18, and 24 days, respectively; estradiol levels were not elevated relative to the control at any sampling time.^[160] A single IV administration of doses as low as 0.5 mg/kg to ovariectomized rats induced prolonged (3–6 weeks) pharmacological effects as measured by LH suppression^[156,160,161] or reduced rate of weight gain.^[156,162-164] It reduced serum testosterone levels in male rats, decreased the growth of the prostate tumor by 61% after two weeks (a larger decrease than that produced by castration),^[165] and re-established copulatory behavior in castrated male rats.^[70]

A single IV administration of estradiol-CDS to cycling female rats caused a dose-dependent inhibition of ovulation that lasted three times longer than with estradiol alone.^[156] In contrast to multiple implants of estradiol-pellet, multiple injections of estradiol-CDS (weekly 1.0 mg/kg injections for 3 weeks)

resulted in significant attenuation of the rise in the tail-skin temperature surges associated with administration of naloxone to morphine-dependent, ovariectomized rats (an animal model for menopausal hot flashes).^[166]

3.3.1. Luteinizing Hormone (LH) and LH-Releasing Hormone

As described earlier, single IV administration of estradiol-CDS doses as low as 0.5 mg/kg to ovariectomized rats induced prolonged (3–6 weeks) pharmacologic effects as measured by LH suppression.^[156,160,161] As shown in Figure 4, estradiol-CDS significantly suppressed LH secretion at doses as low as 0.1 mg/kg through 18 days, while estradiol or estradiol-valerate were ineffective at doses equimolar to the highest examined estradiol-CDS dose (3.0 mg/kg) already at day 12.^[167] Since estradiol-valerate is isolipophilic with estradiol-CDS, this suggests that estradiol-CDS is more than a simple prodrug and can achieve effective targeting and sustained release.

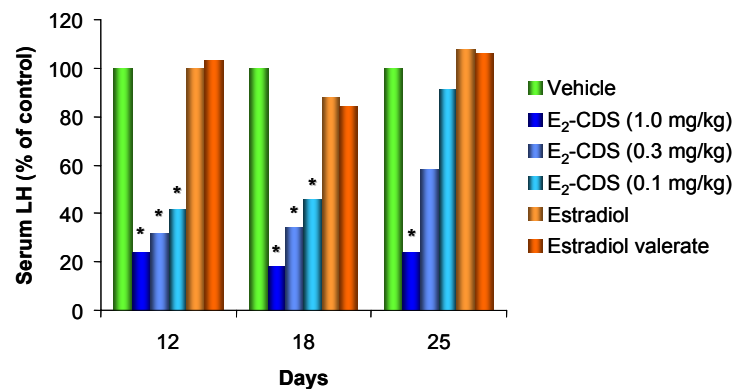


Figure 4. Effects on serum luteinizing hormone (LH) produced by various doses of estradiol chemical delivery system (E₂-CDS) in orchidectomized rats compared with those produced by the vehicle (dimethyl sulfoxide), unsubstituted estradiol, and estradiol valerate. * $p < 0.05$ vs the vehicle (reproduced from Brewster et al.,^[167] with permission).

The effect of estradiol-CDS on LHRH was also studied by Sarkar et al.^[156] Rats that had been ovariectomized for ≥ 2 weeks received a single IV dose of estradiol-CDS (7.7 $\mu\text{mol/kg}$), estradiol (7.7 $\mu\text{mol/kg}$), or vehicle (dimethyl sulfoxide; 1 mL/kg). Only estradiol-CDS significantly reduced pituitary portal plasma LHRH by day 16; a corresponding significant elevation in hypothalamic LHRH suggested tissue accumulation caused by the inhibition of hormone release. Since previous studies^[168,169] have indicated that long-term exposure to estradiol increases pituitary responsiveness to LHRH, these results suggest that the prolonged inhibitory action of estradiol-CDS on LH release is primarily due to sustained suppression of LHRH secretion from the hypothalamus. The reduced LHRH and LH secretion resulted in an abolition of ovulation. A single IV administration of estradiol-CDS to cycling female rats caused a dose-dependent inhibition of ovulation that lasted three times longer than with estradiol alone.^[156]

3.3.2. Male and Female Sexual Dysfunction

It has already been shown that estradiol-CDS can dramatically re-establish copulatory behavior in castrated male rats for at least 35 days, after a single dose.^[70] More recently, a dramatic effect on the sexual behavior of female rats has been noted.^[63] Because FSD is more complex than male sexual dysfunction, such a strong response caused by the administration of an estradiol compound alone was quite unexpected.

The dose and time dependency of the effects of estradiol-CDS, administered as a HP β CD complex, on the re-establishment of copulatory behavior and on plasma LH and estradiol levels in castrated male and female rats has been examined.^[63] Estradiol-CDS re-established copulatory behavior from day 7 to day 28 in orchidectomized males after single IV doses of 0.3 and 3.0 mg/kg. LH suppression started on day 1 and lasted until day 7 and 28, respectively. In ovariectomized female rats, sexual activity was monitored through the lordosis behavior (lordosis being the vertebral dorsiflexion performed by female quadrupeds in response to adequate stimuli from a reproductively competent male). Animals were treated daily for 5 days with IV estradiol-CDS, estradiol benzoate, or vehicle (HP β CD). At a dose of only 0.01 mg/kg, estradiol-CDS already normalized sexual behavior. At the 0.03 mg/kg dose level, the effect estradiol benzoate was 10 days shorter than that of estradiol-CDS. LH suppression lasted for up to 18 days at the 0.03 mg/kg dose, and for up to 10 days at the 0.01 and 0.003 mg/kg doses. On the other hand, there was no significant decrease in LH levels after estradiol benzoate treatment. The low plasma estradiol levels indicated the fast rate of peripheral elimination in both males and females. These results confirm again the potential of estradiol-CDS to cause significantly higher and more prolonged effects than the isolipophilic estradiol benzoate.

3.3.3. Neurodegenerative Models

The effects of estradiol-CDS on neuronal systems known to be dysfunctional in AD were evaluated in an initial study.^[170] Two weeks following ovariectomization, female rats were treated with estradiol-CDS (1 mg/kg, IV) or vehicle (dimethyl sulfoxide), and 5 days later they were sacrificed to determine high-affinity choline uptake in the cortex, hippocampus, and striatum. In the cortex and hippocampus, but not in the striatum, estradiol-CDS treatment increased high-affinity choline uptake to levels that were statistically no different from those of the intact control group. This preliminary, single-dose study indicates that estradiol-CDS can enhance the activity of cholinergic neurons in an ovariectomized animal model.

The effects of estradiol-CDS on neuropeptide Y (NPY) and somatostatin were also briefly evaluated,^[170] since neurons in the brain and/or cerebrospinal fluid of patients with AD show reduced levels of these peptides. Following estradiol-CDS administration, animals were sacrificed at 0 (vehicle – dimethyl

sulfoxide), 1, 7, and 14 days. Tissue samples from the cortex, hypothalamus, hippocampus, striatum, and brain stem were assayed for NPY and somatostatin by specific radioimmunoassays. NPY levels showed a progressive increase in the hypothalamus and cortex, and a transient elevation in the hippocampus; striatal and brain stem NPY levels were not affected. Somatostatin concentrations did not change following this single-dose administration.

3.3.4. Neuroprotection

Estradiol-CDS has also been shown to provide neuroprotective effects. In ovariectomized rats, pretreatment with estradiol-CDS decreased the mortality caused by middle cerebral artery (MCA) occlusion from 65% to 16%.^[129] Even when administered 40 or 90 min after MCA occlusion, estradiol-CDS reduced the area of ischemia by 45–90% or 31%, respectively. Another study provided evidence that treatment with estradiol-CDS can protect cholinergic neurons in the medial septum from lesion-induced degeneration.^[171]

3.4. Toxicology

Extensive safety evaluations have been already conducted on estradiol-CDS by Brewster et al.^[167] Estradiol-CDS formulated in HP β CD was shown to lack mutagenic potential in bacteria, as measured in a standard Ames test.^[167] It was tested in Sprague-Dawley rats and cynomolgus monkeys over a period of either 14 (subacute) or 90 (subchronic) days. In the subacute studies, no effects of the vehicle (HP β CD) were apparent, and the estrogen caused expected changes in hematology (i.e. increased neutrophils) in both species (5 male and 5 female rats, 1 male and 1 female monkeys). In rats, various expected estrogenic reactions were observed (e.g. weight loss and pituitary hypertrophy). In the subchronic paradigm, HP β CD (200 mg/kg) and estradiol-CDS (2.5 mg/kg for 10 male and 10 female rats; and 1.0 and 5.0 mg/kg for 4 male and 4 female monkeys) were administered every other day for 90 days. There was no mortality in the study, and no abnormal cage-side behaviors were observed. Various estrogen-sensitive parameters were significantly altered (decreased body weight, decreased white blood cell count, increased pituitary weight in rats; and decreased protein and glucose count and increased triglycerides in monkeys). The toxicological evaluation of the data indicated that the no observable adverse effect dose was the maximum dose used in the protocol, i.e. 2.5 mg/kg in rats and 5 mg/kg in monkeys.

The neurotoxicologic potency of these compounds was also examined, as this is of special interest for CNS-targeted delivery systems.^[172] Intravenous estradiol-CDS (cumulative doses of 0.2 to 40 mg/kg), saline, or cyclodextrin vehicle was administered to conscious, restrained cynomolgus monkeys (1 male and 1 female) every other day for two weeks. No altered motor activity

was observed during the study, and neurochemical analysis of brain samples performed by HPLC indicated no effect of estradiol-CDS on striatal dopaminergic concentration. These observations suggest the neurotoxicologic safety of these materials.

4. Estradiol-CDS: Clinical Investigations

To date, four phase I/II clinical trials of estradiol-CDS have been completed.^[142,167,173-177] The first study^[173] was designed as a rising dose safety study in postmenopausal women, where plasma LH and follicular stimulating hormone (FSH) could be readily assayed as indicators of pharmacologic action. Volunteers received an IV injection and remained at the clinic for 48 hours. Subjective side effects and vital signs were recorded. Blood samples for LH, FSH, and estradiol analysis were collected at 0.24, 0.5, 1, 2, 4, 8, 24, and 48 hours after drug administration. Additional blood samples were collected after release from the clinic on the mornings of day 4 and 7. All subjects met the criteria for inclusion, and all subjects completed the protocol. No adverse effects that could be attributed to estradiol were reported, and all hematological and clinical chemistry values were unaffected by administration of the drug. Results could be clustered in three dose-groups: 10–40 μg ($n = 3$) showed minimal changes, 80–640 μg ($n = 5$) showed threshold effects, and 1280 μg ($n = 2$) showed substantial and sustained decrease in plasma LH levels (Figure 5). The mean peak decreases obtained for these groups (11%, 34%, and 50%, respectively) compared favorably to those of 28–36% observed after 1 month of dosing with estradiol transdermal patches.

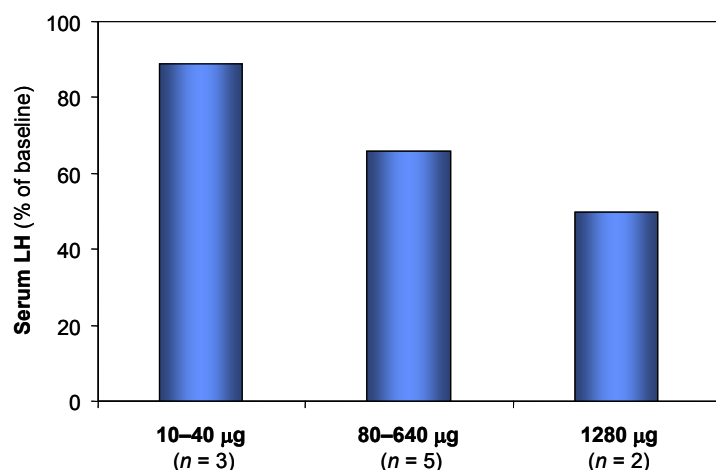


Figure 5. Effect of various grouped doses of estradiol chemical delivery system on mean luteinizing hormone (LH) suppression relative to baseline values in postmenopausal human volunteers (reproduced from Brewster et al,^[167] with permission).

While LH was suppressed in a clinically meaningful way up to 96 hours after estradiol-CDS treatment, levels returned to

control values within 24 hours after estradiol administration. LH suppression induced by estradiol-CDS returned to control values after 168 hours. If the area under the concentration-time curve (AUC) of the LH suppression from 24 to 168 hours is considered (disregarding the initial LH drop common to both treatments), the ratio of estradiol-CDS to estradiol is greater than 10-fold. Additionally, initial serum estradiol levels generated by estradiol-CDS administration are 5- to 10-fold lower than those generated by estradiol administration. This also should provide an increased therapeutic index, especially when estrogens are contraindicated, e.g. because of the possibility of the development of breast cancer.

A second clinical evaluation^[176] was designed to: increase the number of patients exposed to estradiol-CDS, collect further safety information, establish the feasibility of buccal administration, and demonstrate the central action of estradiol-CDS relative to a commercially available oral and parenteral estradiol preparations. A buccal formulation was considered so as to avoid the irreversible acid-catalyzed water addition at the 5,6-double bond of the dihydropyridine targetor moiety, which is unavoidable following traditional oral administration and would render the CDS ineffective for brain targeting. For buccal administration, a comparison with biological effects suggests significant bioavailability. Both IV and buccal administration of estradiol-CDS demonstrated dose-related suppression of circulating LH levels. A comparison of AUCs for LH suppression and plasma estradiol levels demonstrated that for a certain blood level of estradiol, estradiol-CDS is significantly more active than estradiol valerate for both buccal and IV administration. While both formulations exerted similar effects on LH suppression (ratio of 0.7), estradiol-CDS elevated plasma estradiol levels by only a very small fraction (0.044) of that produced by estradiol administration. Furthermore, because of the long half-life of estradiol-CDS in the CNS, an administration regimen can easily be devised to minimize plasma estradiol compared to central estradiol as suggested by a cursory pharmacokinetic evaluation of the data.

A recent pilot phase II study of a newly developed buccal formulation tablet used circulating LH and FSH as biomarkers.^[145,178] This was a single-center, open label study using 12 healthy postmenopausal female volunteers who were not on estrogen therapy. Volunteer were divided into two sequential groups (with 6 per group) receiving one or two buccal tablets. Tablets administered on the first day of the study contained placebo, and each tablet administered on the second day contained estradiol-CDS 2.5 mg. Plasma levels were monitored for the following hormones: LH, FSH, estradiol, prolactin, and estrone.

Following a single dose of estradiol-CDS, sustained plasma estradiol levels were maintained for up to 3 days, confirming the release of active estradiol from the intermediate ‘locked in’ the

brain. Additionally, peak plasma estradiol levels were considerably lower than those expected to be produced by administration of an equivalent dose of estradiol, and they were within, or just slightly above, the normal premenopausal fluctuating range of 20–250 pg/mL. AUC data also showed a clear dose-response.

Both LH and FSH blood levels were suppressed in a statistically significant manner (paired *t*-test analysis) (Figure 6) as also indicated by the AUC to 24 hours (AUC₂₄) data.^[145,178] The study, therefore, confirmed the potential of estradiol-CDS to deliver brain-targeted estradiol, producing CNS-related activities and providing, at the same time, prolonged, sustained release, resulting in safe, but sufficiently elevated peripheral hormone levels.

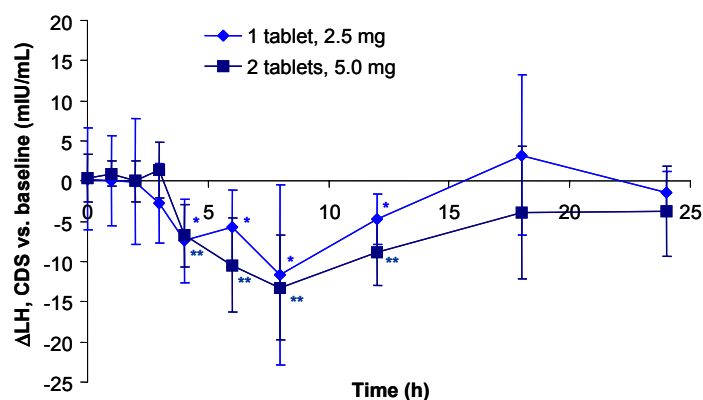


Figure 6. Luteinizing hormone (LH)-suppression in postmenopausal volunteers caused by estradiol chemical delivery system administered as one or two 2.5 mg buccal tablets on the second day of the study; tablets administered on the first day contained placebo.^[145,178] Blood level data shown as mean \pm standard deviation for 6 subjects per group; change in LH calculated as treatment (day 2) levels minus baseline (day 1) levels for each subject. * $p < 0.05$, ** $p < 0.01$ vs baseline using paired *t*-test.

Another recent phase Ib/II study with the buccal tablet has also been completed.^[145,179] This was a single-center, open-label, parallel-group study in 12 healthy postmenopausal females on no estrogen or hormonal therapy. They were divided into two groups of 6 and received one buccal tablet containing estradiol-CDS 2.86 mg once daily for 10 days (group A) or once every second day for 13 days (group B). Blood and urine samples were collected and assayed for total and free estradiol, estrone, testosterone, LH, FSH, sex hormone binding globulin, and prolactin. Urine levels of 2-hydroxyestrone (2OHE₁) and 16 α -hydroxyestrone (16OHE₁) were also monitored. The corresponding pharmacokinetic/pharmacodynamic parameters were calculated.

In this study, buccal estradiol-CDS administered daily or every other day was well-tolerated. Serum estradiol levels were higher for the daily regimen (group A), and differences were significant (Figure 7).^[145,179] Both regimens caused significant

LH- and FSH-suppression, providing evidence of CNS penetration. Effects on LH and FSH were observed at low serum levels of estradiol. FSH-response was still evident 3 days after the last dose of estradiol-CDS, when serum estradiol levels were near baseline. The 2OHE₁/16OHE₁ ratio was increased by estradiol-CDS, indicating a potential low risk for breast cancer.

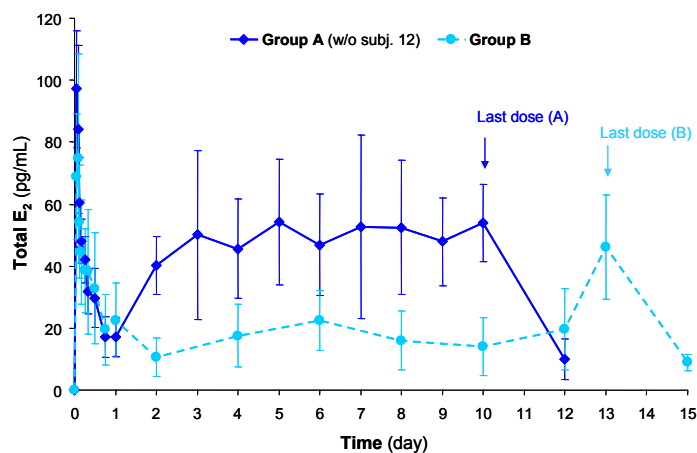


Figure 7. Baseline-adjusted total estradiol concentrations following administration of estradiol chemical delivery system 2.86 mg as a buccal tablet once daily (group A) or once every other day (group B).^[145,179] Data are mean \pm standard deviation for 6 female volunteers in group B and 5 female volunteers in group A. Data from one subject in group A, who had ~10-fold higher estradiol levels compared to other subjects, were excluded from the graph.

5. Conclusions

Brain-targeted estradiol delivery could be a useful and safe therapy for menopausal symptoms including hot flashes in general, and estrogen-dependent cognitive deficits, AD, male and female sexual dysfunction, and neuroprotection in particular. A CDS approach has been demonstrated to be a useful, potent, and long-lasting estradiol delivery system that targets the brain and CNS. Clinical evaluations suggested a potent central effect coupled with only marginal tendencies to elevate systemic estrogen levels. Furthermore, peripheral levels seem to be susceptible to easy manipulation through dosage adjustments and frequency of administration; therefore, such an approach could represent the ideal estrogen therapy.

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