

Applications of chemically-modified cyclodextrins: use of hydroxypropyl- β -cyclodextrin as an enabling excipient for brain targeting, redox-based derivatives of estradiol A review of preclinical and clinical findings

M.E. Brewster^{1*}, T. Loftsson², N. Bodor³

¹Department of Pharmaceutical Sciences, Johnson & Johnson Pharmaceutical Research and Development, Beerse, Belgium

²Faculty of Pharmacy, University of Iceland, Reykjavik, Iceland

³Ivax Corp., Miami, FL, United States, and Ivax Drug Research Institute, Budapest, Hungary

*Correspondence: mbrewste@prdbe.jnj.com

Estrogen depletion associated with the menopause produces a constellation of debilitating symptoms which range the gamut from vasomotor complaints which severely affect over one-third of all climacteric women to cognitive deficits. While many of these complications can be alleviated with traditional hormone replacement therapy (HRT), fear of cancer, cardiovascular problems and other metabolic diseases prompt many women to avoid treatment. Surveys suggest that over 30% of all prescriptions written for HRT are never filled due to these concerns and recent findings from Woman's Health Initiative trials have only added to these trepidations. Selective brain delivery of estrogens, in general, and estradiol, in particular, may address these items. One approach to accomplish CNS deposition is the use of a chemical delivery system for estradiol that selectively targets the central nervous system and may, therefore, reduce the incidence or severity of peripherally manifested side-effects. Owing to the very poor water solubility and limited stability of these derivatives, hydroxypropyl- β -cyclodextrin has proved to be enabling excipients in the development of these drug candidates. Various preclinical evaluations have demonstrated the organ-targeting potential of an estradiol-chemical delivery systems (E2-CDS) and therefore its potential usefulness as a therapeutic adjunct in certain subpopulations of menopausal women including those at risk to breast carcinomas. A very exciting potential use of the E2-CDS is in neurodegenerative diseases. Data suggest that estrogens may improve the mental performance of elderly patents suspected of having Alzheimer's disease.

Key words: Chemical delivery systems (CDS) – Estradiol – Hydroxypropyl- β -cyclodextrin.

I. INTRODUCTION

A variety of approaches have been applied to the delivery of drugs to the brain including physical methods in which the drug is either i) injected into the CSF or brain parenchyma, ii) surgically placed at its site of action using various biodegradable matrices or iii) introduced into the brain through a temporary breakdown of the blood-brain barrier (BBB) which is induced by carotid injections of hypertonic nonelectrolyte solutions [1-10]. Biological approaches have explored the use of macromolecular carriers such as chimeric peptides or antibodies [11-13]. Chemical methods have taken the form of drug analogs and prodrugs [14-17]. A prodrug is a pharmacologically inactive compound which results from transient chemical modification of a biologically active species [18]. The chemical change is designed to improve some deficient physicochemical property of the drug such as membrane permeability or water solubility. When the BBB is considered, increased drug penetration is usually well correlated with the lipophilicity or the octanol: water partition coefficient of a drug [14, 19]. In order, therefore, to improve the entry of drug, esterification or amidation may be performed. This manipulation provides the necessary lipid solubility of the drug and improves BBB penetration. Once in the CNS, hydrolysis of the lipophilicity-modifying group will release the active compound [18].

Chemical delivery systems

Unfortunately simple prodrugs suffer from several important limitations, the most severe of which is nonselectivity in target

site delivery. In addition, poor retention and the possibility for reactive catabolism, often act to decrease, not increase, the therapeutic index of drugs when masked as a prodrug. Some of the limitations associated with the prodrug approach are derived from the fact that only one chemical conversion occurs in the activation of the compound. In many circumstances, multiple and facile conversions may not only lead to selectivity in delivery but may also act to decrease the toxicity of a drug as well as sustain its action. A chemical delivery system (CDS) is defined as a biologically inert molecule which requires several steps in its conversion to the active drug which enhances drug delivery to a particular organ or site [20-22]. In designing a CDS for the CNS, the unique architecture of the BBB was exploited. As with a prodrug, a CDS should be sufficiently lipophilic to allow for brain uptake. Subsequent to this step, the molecule should undergo an enzymatic or other conversion to promote retention within the CNS but, at the same time, to accelerate peripheral elimination of the entity [23]. Finally, the intermediate should degrade releasing the active compound in a sustained manner. One system which possesses these attributes is summarized in *Figure 1*. In this CDS, a targetor molecule is used as a lipophilicity modifier. While many moieties may serve such functions, 1,4-dihydronicotinates or trigonellinates have proved to be the most useful. In this approach, a hydroxy-, amino- or carboxylic acid-containing drug is esterified, amidated or otherwise covalently linked to nicotinic acid or a nicotinic acid targetor precursor. This compound is then quaternized to generate the 1-methylnicotinate salt or trigonellinate and chemically reduced

to give the 1,4-dihydrotrigonellinate or CDS. Upon systemic administration, the CDS can partition into several body compartments due to its enhanced lipophilicity; some of which are inaccessible to the unmanipulated compound. At this point in the delivery scheme, the CDS is simply working as a lipoidal prodrug. The carrier molecule is specially designed, however, to undergo an enzymatically mediated oxidation which converts the membrane permeable dihydrotrigonellinate to a hydrophilic, membrane impermeable trigonellinate salt [23].

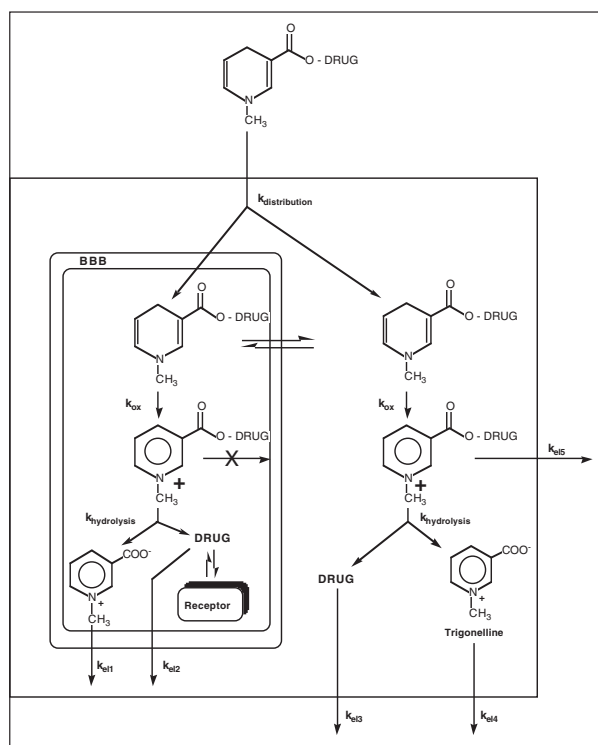


Figure 1.

This conversion occurs ubiquitously. The now polar, oxidized carrier-drug conjugate is trapped behind the lipoidal BBB and is, in essence, depoted in the CNS. Any of the oxidized salt which is present in the periphery will be rapidly lost as it is now polar and easily eliminated by the kidney and liver. The conjugate which is trapped behind the BBB can then slowly hydrolyze to give the active species in a slow and sustained manner. By the system design, concentrations of the active drug are very low in the periphery reducing systemic, dose-related toxicities. In addition, the drug in the CNS is present mostly in the form of an inactive conjugate, thus lowering any central toxicities. This approach should allow for a more potent compound in that a larger portion of the administered dose is shunted to its site of action. In addition, this system should allow for an increased dosing interval. The brain-targeting CDS has been extensively applied to various pharmacologically active agents [24-30]. Several excellent reviews of this technology are available [31-33].

II. AN ESTRADIOL-BASED CHEMICAL DELIVERY SYSTEM (E2-CDS)

The most advanced CDS is a brain-targeting system for es-

tradiol. Estrogens are lipophilic steroids which are not impeded in their entry to the CNS. These compounds readily penetrate the BBB and achieve high central levels after peripheral administration. Unfortunately, estrogens are poorly retained by the brain. This circumstance requires that frequent doses of these steroids be administered to maintain therapeutically significant concentrations. Constant peripheral exposure of estrogen has been related, however, to a number of pathological conditions including cancer, hypertension, and altered metabolism in addition to uterine bleeding and other complications [34-40]. As the CNS is the target site for many of the actions of estrogens, a brain-targeted delivery form of these compounds may provide for safer and more effective estrogens [41, 42].

A broad spectrum of potential clinical applications for estrogen chemical delivery systems is available (Table I) and this reflects the wide distribution of estrogen receptors in the brain. Estrogen receptors are found in the hypothalamus and the closely associated preoptic area, where they mediate estradiol effects on LH secretion, sexual behavior in both males and females, appetite and temperature regulation [43, 44]. In addition, estrogen receptors have recently been identified on dopaminergic neurons which innervate the striatum, where estradiol may modulate locomotion and hence may be involved in movement disorders [45]. Also estrogen receptors have been identified in mesocortical dopaminergic neurons where they may mediate estradiol effects on mood [46]. Finally, estrogen receptors are found in the region of the nucleus basalis magnocellularis (NMB), a site of cholinergic cell bodies which innervate the cerebral cortex [47]. These estrogen receptors may mediate the influence of estrogens on cognitive function.

Table I - Potential Indications for an estradiol chemical delivery system.

- Menopausal symptoms
- Prostate cancer
- Endometriosis
- Libido
- Cognition and Alzheimer's disease
- Depression
- Contraception

1. Chemistry

The most potent natural steroid is estradiol (E2), which is chemically a diol containing a phenolic 3-moiety and a 17-alcohol group. The preparation of the 17-based CDS is given in Figure 2. This manipulation, in addition to generating a CDS, efficiently destroys the biological potency of the estrogen since 17-substituted estradiol analogs are known not to bind to the estrogen receptor [48]. In the synthesis, E2 is reacted with nicotinoyl chloride hydrochloride to give the 3,17-bis nicotinate [49-51]. This compound is subjected to methanolic potassium bicarbonate which results in selective hydrolysis of the phenolic nicotinate. The resulting secondary ester is quaternized with methyl iodide to give the 17-trigonellinate, or E2Q⁺, and then reduced using sodium dithionite to give the 17-(1,4-dihydrotrigonellinate), or E2-CDS. This synthesis has been scaled to large batch size high yield and purity. The log P values for the E2-CDS and E2-Q⁺ were measured using standard procedures and compared to that of E2. The log of the octanol:

water partition coefficient for E2-CDS was found to be 4.50; for E2, 3.76; and for the E2-Q⁺ salt, 0.144 [49]. This indicates that the quaternary salt form of E2-CDS is 8000 times more hydrophilic than the parent E2 and more than 44,000 times more hydrophilic than the E2-CDS which is approximately five-fold more lipophilic than E2. These values, which are consistent with other derivatives examined, indicate that the compounds synthesized possess the appropriate physicochemical properties for CDS functioning.

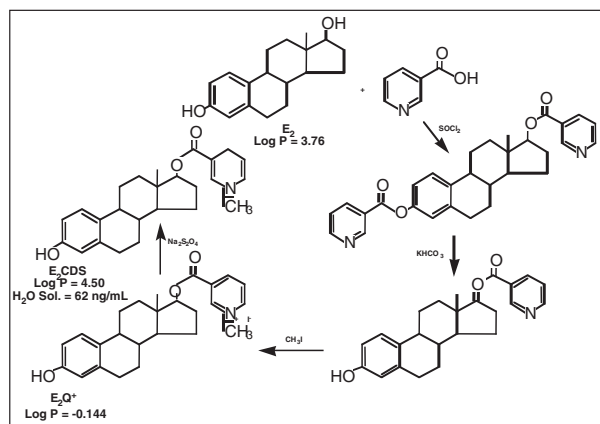


Figure 2 - Preparation of E2-CDS (3-hydroxy-17 β -[(1-methyl-1,4-dihydropyridin-3-yl)-carbonyl]oxy]estra-1,3,5(10)-triene), E2-Q⁺ (1-methyl-3-[[[3-hydroxyestra-1,3,5(10)-triene-17 β -yl]oxy]-carbonyl]pyridinium iodide), estradiol nicotinate (3-hydroxy-17 β -[(3-pyridinylcarbonyl)oxy]estra-1,3,5(10)-triene and estradiol bis nicotinate (3, 17 β -bis[(3-pyridinylcarbonyl)oxy]estra-1,3,5(10)-triene).

2. Formulation

The E2-CDS is designed to penetrate into deep brain compartments and to be biologically labile to generate the requisite *in vivo* conversions. These properties complicate the development of a convenient formulation due to the poor water solubility of the E2-CDS as well as its hydrolytic and oxidative instabilities [52]. This problem was overcome through the use of chemically modified β -cyclodextrins, specifically 2-hydroxypropyl- β -cyclodextrin. These derivatives interact with lipophiles resulting in the formation of a dynamic inclusion complex [53-55]. These complexes act to both increase the water solubility of the E2-CDS (from about 50 ng/ml to over 30 mg/ml) as well as to significantly increase its shelf-life (Figure 3) [52]. While a number of the initial studies for E2-CDS were completed using organic vehicles such as DMSO, the toxicological and clinical development of the compound would not have been possible without the use of the functional excipient, HP β CD.

3. In vitro studies

The CDS concept requires that the dihydrotrigonellinate-drug conjugate rapidly converts to the pyridinium salt and that this brain lock-in form slowly hydrolyzes to give the parent drug and the carrier molecule (trigonelline). Prior to *in vivo* study, these assumptions were examined *in vitro* using rat organ homogenates as the test matrices. The half-lives of E2-CDS in plasma, liver, and brain homogenates were found to be 156.6,

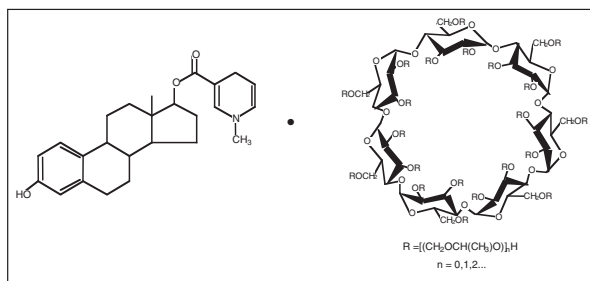


Figure 3 - HP β CD complex with E2-CDS.

29.9, and 29.2 min, respectively [49]. Thus, E2-CDS was converted to the corresponding pyridinium salts much faster in the tissue homogenates than in plasma. This is consistent with a membrane-bound enzyme acting as the oxidative catalyst. Candidates for this enzyme include members of the family of NADH transhydrogenases which mediate redox reactions of NADH, a molecule similar in structure to the active portion of the CDS [23]. The second metabolic step required for drug release is hydrolysis of E2 from the E2-Q⁺ which was demonstrated in plasma as well as in various organ homogenates.

4. Tissue distribution and pharmacokinetics

Initial studies on the tissue distribution of E2-CDS involved i.v. administration of large doses of the drug dissolved in DMSO (60 or 15 mg/kg) to conscious restrained rats [49, 65]. The E2-CDS rapidly disappeared from blood after administration reflecting the enzymatic lability and large volume of distribution of this lipophile. The quaternary metabolite of E2-CDS was detected in all tissues soon after drug administration. Analytical determinations were assisted with the development of a pre-column-enriching HPLC system [65]. This method generated limits of accurate detection for plasma samples and organ tissue of 10 ng/ml or ng/g for E2-Q⁺, 20 ng/ml or ng/g for the E2-CDS and 50 ng/ml or ng/g for E2. As illustrated in Figure 4, a significant selectivity was observed for the CNS with tissue half-lives being approximately five-fold greater than those observed in kidney, heart, lung, testis, eye, or peritoneal fat [65].

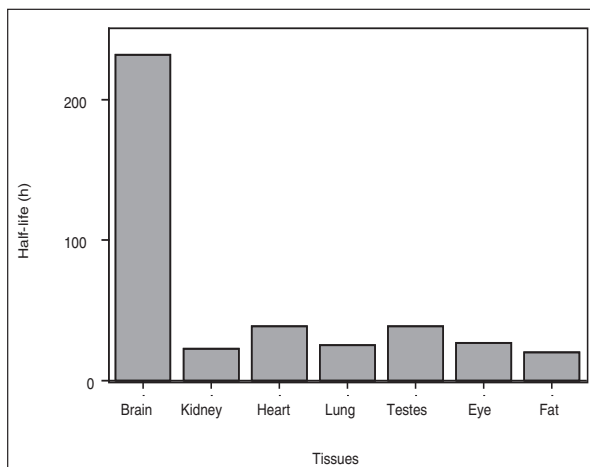


Figure 4 - Elimination half-lives for E2-Q⁺ from various tissues.

In order to follow E2 released over time from deposited E2-Q⁺, an RIA technique was developed [57]. The levels of E2 in brain tissue after E2-CDS administration are elevated four to five times longer than after simple E2 treatment (Figure 5). In addition, the ratio of the area under the concentration curves (AUC) of E2 in brain and plasma after E2-CDS treatment was approximately 12. This profile confirms that E2 is generated within the CNS and is not sequestered from peripheral sources. In addition, plasma levels of E2 are much more rapidly eliminated than those in brain (Figure 6). Pharmacokinetically, serum E2 data derived from E2-CDS dosing could be fitted to a three exponent time-concentration curve in which the initial phase (half-life = 1.0 h) was correlated with a rapid distribution phase, the second phase was associated with elimination of E2 derived from several compartments (both peripheral and central) (half-life = 9.9 h) and the terminal elimination phase (half-life = 123.5 h) was associated primarily with elimination of E2 derived from the brain [57]. The pharmacokinetics of the E2-CDS have also been examined in the dog [58]. The data collected from these evaluations confirm the large volume of distribution for the E2-CDS as well as its dose linearity and slow terminal phase elimination (Table II).

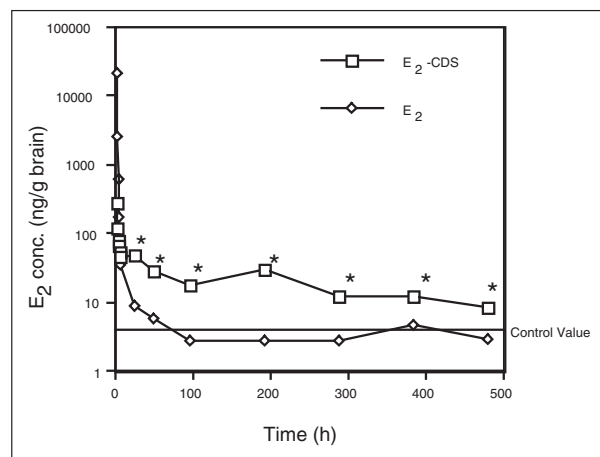


Figure 5 - Brain E2 after E2-CDS (3 mg/kg) or equimolar E2.

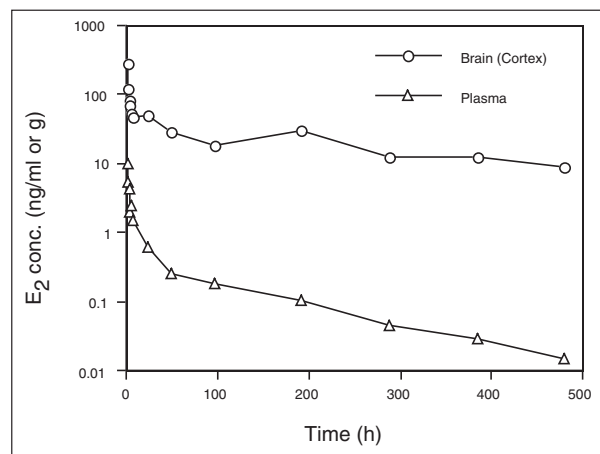


Figure 6 - Brain (cortex) and plasma E2 after a single 3 mg/kg dose of E2-CDS.

Table II - Pharmacokinetics of E2-CDS in the dog.

- E2-CDS (1 mg/kg)
 - $Cl_{total} = 11.9 \pm 4.7$ l/min
 - $MRT = 23 \pm 11$ min
 - $Vd_{ss} = 286$ l (12 l/kg)
- E2-Q⁺
 - Protein binding = 87%
 - $AUC = 291 \pm 23$ ng·h/ml (0.5 mg/kg) and 451 ± 20 ng·h/ml (1.0 mg/kg)
 - $Cl_{renal} = 37.1 \pm 1.8$ ml/min
 - $k_e = 0.315$ d⁻¹ ($t_{1/2} = 2.2$ d)

5. Pharmacology

Given this kinetic advantage, the pharmacology of the estradiol delivery system was examined using a variety of animal models including the effects of the system on i) LH and LHRH secretion, ii) cyclicity and ovulation in the rat, iii) sexual behavior in castrate male rats, iv) testosterone levels and androgen-dependent peripheral tissues, v) body weight and food intake, vi) growth hormone dynamics and other hormonal effects and vii) high affinity choline uptake, neuropeptide Y and cerebral ischemia.

Estradiol, in the ovariectomized female, acts in a negative feedback mode to decrease the secretion of LHRH at the levels of the hypothalamus [59]. The hormonal regulation further acts to reduce LH secretion at the pituitary which then reduces E2 production at the level of the ovaries (Figure 7) [60]. It is generally acknowledged that E2 can affect several behaviors at higher brain centers including weight maintenance, cognition, thermoregulation and depression meaning that the estradiol depletion associated with ovarian removal or senescence may alter or complicate these activities [41, 42]. The initial examinations of the E2-CDS were aimed to evaluating its effect on the hypothalamic-pituitary-ovarian axis.

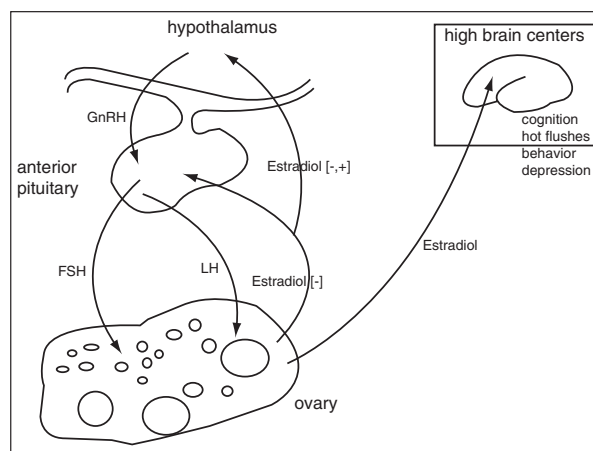


Figure 7 - Estradiol dynamics in the female.

5.1. LH and LHRH

The response of LH secretion to E2-CDS was evaluated in several rat studies. In the first set of experiments, E2-CDS was administered as a single i.v. injection (3 mg/kg) in DMSO to male rats which had been orchidectomized 2 weeks previously [61, 62]. E2 was administered to another

group of rats at an equimolar dose and vehicle-treated rats served as the controls. Both E2 and E2-CDS reduced serum LH equivalently from 4 to 48 h post-drug administration. From 4 to 12 days, LH levels in the E2 treated rats increased progressively to levels equivalent to those in DMSO-treated rats (Figure 8). By contrast, LH concentrations in animals treated with E2-CDS remained low and were suppressed by 82%, 88% and 90% when compared to vehicle treated animals at 4, 8 and 12 days after treatment, respectively. In these animals, serum E2 levels remained elevated through 2 days in both rats treated with E2 and E2-CDS. By 4 days after drug treatment, E2 levels in both drug-treated groups returned to that observed in vehicle treated orchidectomized male rats. These data indicated that, despite the clearance of E2 from the plasma, the delivery system achieved chronic suppression of LH secretion for at least 12 days following a single i.v. administration. These data are consistent with the hypothesis that the E2-CDS causes enhanced brain delivery, lock-in of the E2-Q⁺, and slow release of E2 to achieve LH suppression. This study was extended to determine the duration of LH suppression following several different doses of the E2-CDS. In this second evaluation, orchidectomized rats were treated with E2-CDS solubilized in DMSO (0.1, 0.3 or 1.0 mg/kg i.v.) or the vehicle and were examined at 12, 18 and 24 days post-treatment [50, 63]. E2-CDS significantly suppressed LH secretion at the lowest dose through 18 days while estradiol or estradiol valerate (E2V, a depot ester of estradiol) were ineffective at doses equimolar to the highest E2-CDS dose at 12 days (Figure 9).

The secretion of LH in rats can be controlled or attenuated by numerous factors, including the action of E2 both in a positive and negative feedback mode at the pituitary gland. In addition, hypothalamic release of LHRH stimulates LH secretion. Again, LHRH release can be augmented by the interaction of E2 in the preoptic area. The effect of E2-CDS on LHRH release was, therefore, studied [57]. Animals which had been ovariectomized for at least 2 weeks were given a single i.v. dose of E2-CDS, E2 or vehicle. At either 1 or 16 days post-drug administration, animals were anesthetized and their pituitary stalks exposed for collection of portal blood samples to be used for LHRH RIA. Subsequent to sample collections, hypothalamic tissue was removed for LHRH assay. Portal blood concentrations of LHRH were significantly reduced on day 1 for both E2 and E2-CDS. By day 16, however, only the E2-CDS continued to decrease LHRH secretion, while the E2 treated animals were not different from vehicle controls (Figure 10). The decreased LHRH secretion was mirrored by an increase in hypothalamic LHRH levels, suggesting that the inhibition of hormone release resulted in a tissue accumulation of the peptide. Again, hypothalamic LHRH concentrations were significantly elevated at day 1 for both E2-CDS and E2, but by 16 days only the E2-CDS produced a significant accumulation (Figure 10). Since previous studies have indicated that long-term exposure of E2 decreases pituitary responsiveness to LHRH, the generated results strongly suggest that the prolonged inhibitory action of E2-CDS on LH release is due primarily to sustained suppression of LHRH secretion from the hypothalamus [64, 65]. The reduced LHRH and LH secretion resulted in an abolition of ovulation as indicated by an

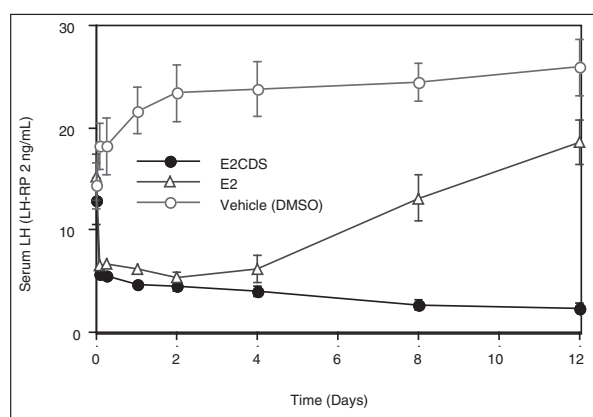


Figure 8 - Serum LH following single doses of E2-CDS (3 mg/kg) or E2 formulated in DMSO to castrate male rats.

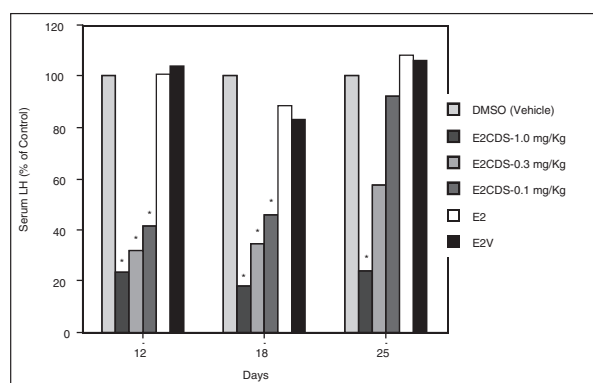


Figure 9 - Dose and duration effects of E2-CDS on LH suppression.

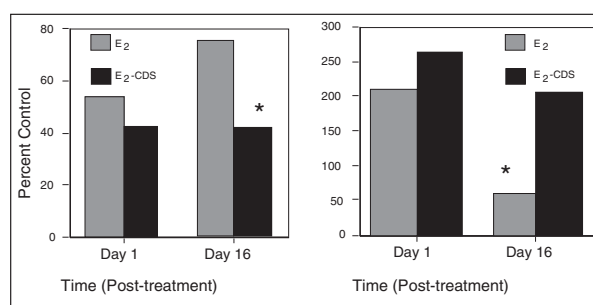


Figure 10 - Effect of E2 or E2-CDS on portal plasma (Left) or hypothalamic (right) LHRH.

elimination of ova found in the oviducts as well as by an increase in the normal ovulation cycle. The increase in the estrus cycle was dose-related and significantly more sensitive to E2-CDS as compared to equimolar E2 (Figure 11). While the pharmacokinetic data demonstrated that peripheral estradiol was not elevated compared to E2 treatment, a biological end point was examined to verify this finding. Treatment of intact female rats with E2-CDS resulted in no change in uterine weight consistent with the failure of the E2-CDS to significantly elevate serum E2. On the other hand, equimolar E2 provided for significant uterine ballooning consistent with the expected elevation in serum E2 (Figure 12).

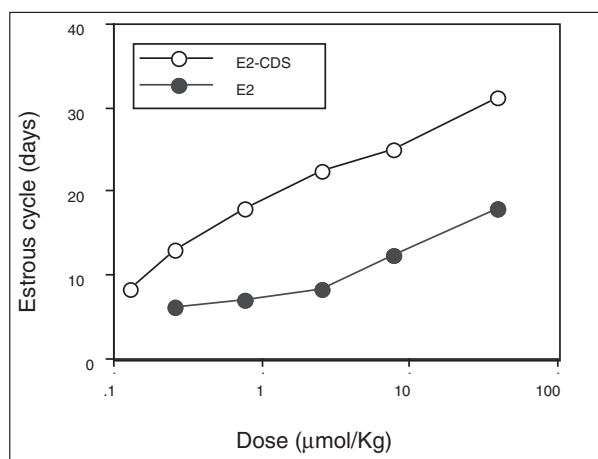


Figure 11 - Effect of E2-CDS or E2 on cycling time in rats.

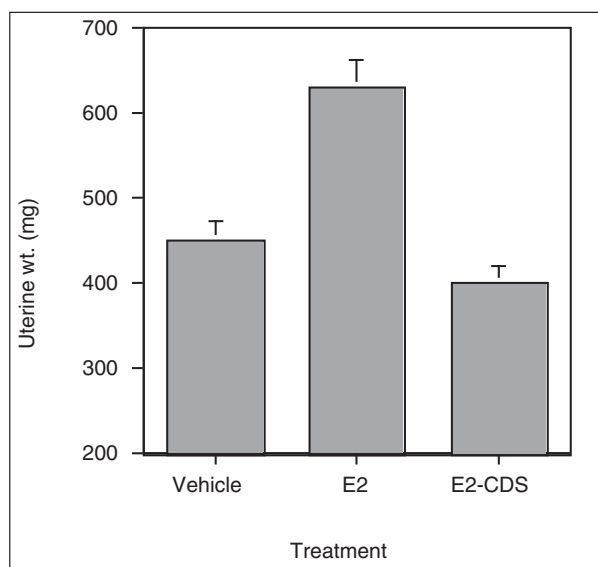


Figure 12 - Effect of E2 or E2-CDS on uterine weight in intact female rats.

5.2. E2-CDS in the male

In the male, E2 can also modulate a variety of biological functions [66-68]. As in the female, E2 can interact at the hypothalamus to reduce LHRH secretion which, in turn, can reduce LH release from the pituitary (Figure 13). The reduced circulating LH concentrations can then attenuate steroidogenesis at the level of the Leydig cells resulting in a reduction in serum testosterone. The reduced serum testosterone can then induce atrophy and programmed cell death at the level of the seminal vesicles and prostate gland, two androgen-dependent peripheral tissues. In addition, estradiol can stimulate sexual behavior at higher brain centers in the male, a supposition confirmed by E2 implant studies [66-68].

5.3. Sexual behavior

The effect of E2 on sexual behavior in castrated male rats was examined [69]. Castration, as expected, has an inhibitory effect on the ability of a male rat to pursue, mount and intromit

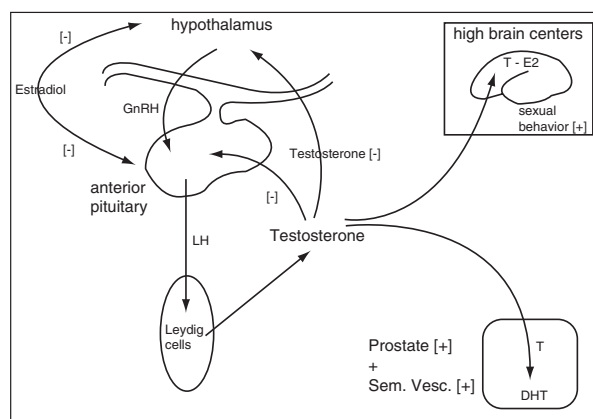


Figure 13 - Estradiol dynamics in the male.

with a receptive female. While E2-CDS and E2V both acted to increase the mounting response at early times, the E2-CDS significantly increased mounting behavior to intact levels (100% responders) through the 35 day time course of the experiment (Figure 14) [69]. The E2-CDS was more potent than either the vehicle control or E2V. Intromission behavior was also significantly increased subsequent to E2-CDS dosing through at least 26 days and tended to increase the behavior through 35 days.

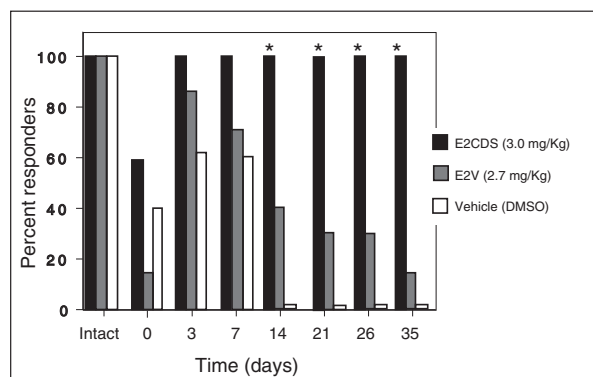


Figure 14 - Effect of E2-CDS or E2V solubilized in DMSO on mounting behavior

5.4. Testosterone and androgen-dependent tissues

The effect of E2-CDS solubilized in HP β CD on serum testosterone in the male rat was illustrated in a number of studies (Figure 15) [70, 71]. Administration of E2-CDS provided for a reduction in serum testosterone levels that was significantly greater than that produced by estradiol benzoate (E2B, a depot ester of E2). The reduction in serum testosterone exacted a diminution in prostate and seminal vesicle weights. Thus, while both E2-CDS and E2V reduced the size of the prostate to castrate levels, the E2V significantly elevated serum E2 levels while the serum levels after E2-CDS were not significantly different from vehicle-treated animals (Figure 16).

5.5. Body weight

E2-CDS was also examined as to its effects on body weight and food intake [72-75]. E2-CDS was significantly more potent in reducing the rate of weight gain in castrate female rats as

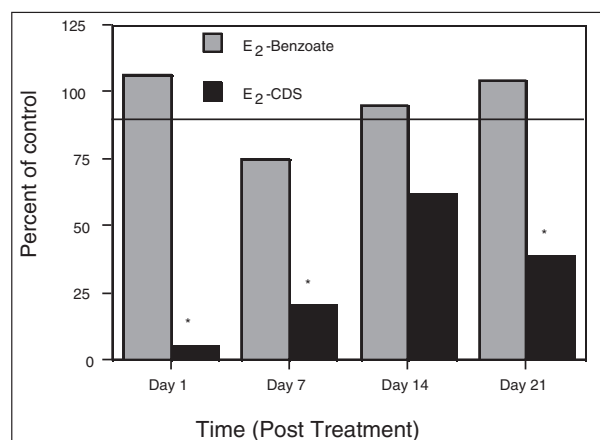


Figure 15 - Effect of E2-CDS (1.0 mg/kg) or E2B solubilized in HP β CD on serum testosterone.

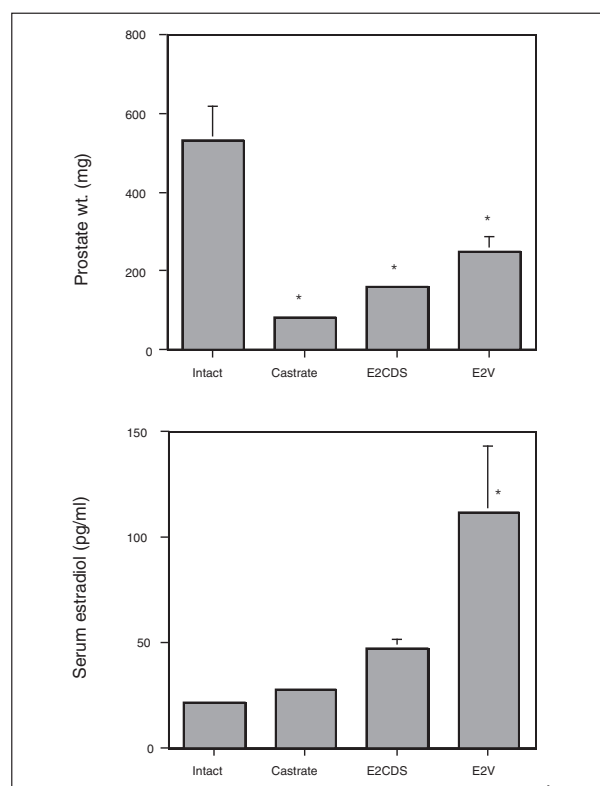


Figure 16 - Effect of E2-CDS or E2V on prostate weight and serum E2 in the rat.

compared to E2V. These differences were significant through five weeks after a single 3 mg/kg administration and tended to reduce body weight through the 100 day time course of the experiment (Figure 17) [75]. In other experiments, 0.3 mg/kg of E2-CDS significantly reduced the rate of weight gain in castrate rats 25 days subsequent to a single dose, while E2V or E2 at doses as high as 1.0 mg/kg exerted no significant activity in this regard at 12 days. Body weight effects have been documented in numerous systems including intact and castrate as well as in lean and obese animals (Table III).

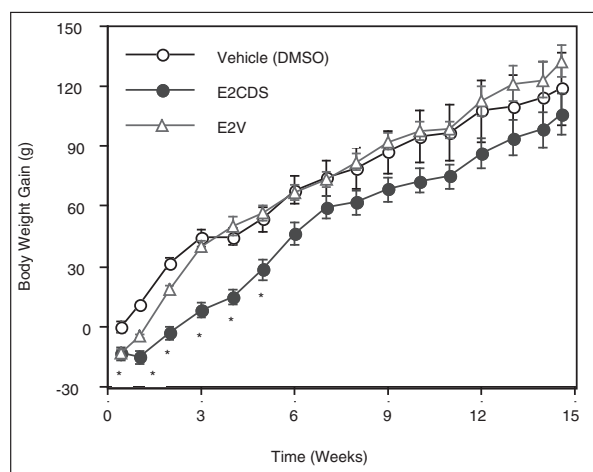


Figure 17 - Effect of E2-CDS or E2V solubilized in DMSO on weight gain in castrate female rats.

Table III - Effect of E2-CDS on body weight in rats.

- Intact females
 - Decreased body weight relative to controls through day 36 (15 mg/kg), day 7 (5 mg/kg), day 3 (1.0 mg/kg) and day 2 (0.2 mg/kg)
- Castrate Females
 - Decreased body weight relative to controls through day 28 (1.0 mg/kg) and day 12 (0.1 mg/kg)
 - Decreased food intake through day 8 (1.0 mg/kg)
- Intact Males
 - Decreased body weights relative to control through day 39 (5 mg/kg and 1.0 mg/kg) and day 14 (0.2 mg/kg)
 - In obese rats, body weight was decreased through day 64 (5 mg/kg), day 50 (1.0 mg/kg) and day 29 (0.2 mg/kg)
- Castrate males
 - Decreased body weight relative to controls through day 24 (3.0 mg/kg)
 - In obese (middle-aged) rats, body weight was decreased through day 14 (1 mg/kg)

5.6. Effect on other hormones

Other pharmacological assays in which E2-CDS has been reported to be more potent than E2 include effects on growth hormone secretion dynamics, prolactin and T₃ (Table IV) [76-78].

Table IV - Other effects of E2-CDS.

- Prolactin - E2-CDS consistently increases prolactin levels in both males and female rats
- Growth hormone (adult, male rats) - E2-CDS (1.0 mg/kg) but not equimolar E2 or vehicle disrupts GH secretory patterns and increases baseline levels
- T₃ (middle aged male rats) - E2-CDS reduced T₃ by 35% at day 7 and 41% by day 14

5.7. Neurodegenerative models

Recent suggestions that E2 may be useful in neurodegenerative disease also prompted an evaluation of pharmacological models in this area [79-83]. The biological basis for the involvement of estrogens in cognition is derived from both laboratory

animal and human data. Estrogen receptors have been observed in the regions of the nuclei of the basal forebrain which are the major loci of cell bodies of cholinergic neurons. These fibers innervate the cerebral cortex, limbic system and hippocampus and these specific brain regions are believed to be involved in the pathology of Alzheimer's disease. In rats, cholinergic neurons respond to peripheral administration of estrogen by increasing the activity of choline acetyl transferase, acetyl choline synthesis and high affinity choline uptake. Estrogens may, therefore, serve in a modulatory role in the function of cholinergic neurons and may serve as therapeutic agents in the stimulation of brain cholinergic neurons in patients with Alzheimer's disease and other dementia which may be due to a cholinergic deficit [84-86]. Given the aforementioned evidence for an effect of estrogen on cholinergic neuronal function, the effects of E2-CDS on neuronal systems which are known to be dysfunctional in Alzheimer's disease were evaluated. In an initial study, female rats were ovariectomized (or received sham surgery) and 2 weeks later the ovariectomized rats were treated with the drug vehicle or E2-CDS (1 mg/kg, i.v.) and 5 days later all animals were sacrificed for a determination of high affinity choline uptake in the cerebral cortex, hippocampus and striatum. Ovariectomy reduced high affinity choline uptake in all three regions evaluated (Figure 18) [87, 88]. Treatment with E2-CDS increased high affinity choline uptake in the cortex and hippocampus to levels which were not different than intact control values. This single dose study, while preliminary, indicates that E2-CDS can enhance the activity of cholinergic neurons in an ovariectomized animal model.

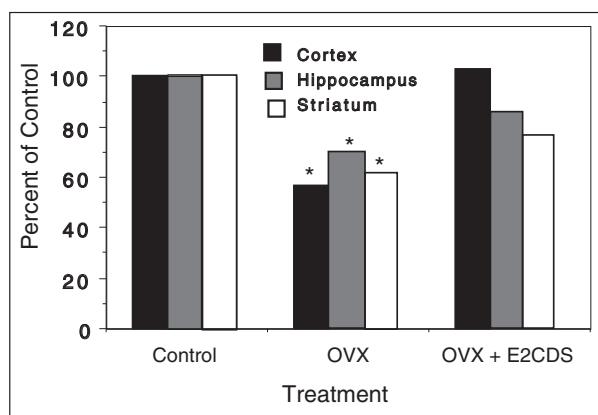


Figure 18 - Effect of E2-CDS (1 mg/kg i.v.) solubilized in HP β CD on high affinity choline uptake in the female rat.

Neuropeptide Y and somatostatin neurons show reduced peptide levels in the brain and/or CSF of patients with Alzheimer's disease [88]. To determine the effects of estradiol on levels of these two peptides, E2-CDS was administered and animals sacrificed at 0 (vehicle), 1, 7 or 14 days later. Tissue samples were obtained from the cortex, hypothalamus, hippocampus, striatum and brain stem and samples were assayed for NPY and somatostatin by specific RIA. In response to E2-CDS, 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 by the E2-CDS. In

contrast to the effects of E2-CDS on NPY levels, somatostatin concentrations did not change in response to administration of a single dose of the delivery system.

The ability of E2-CDS to alter the effects of midcerebral artery occlusion (MCAO) in rats was also assessed in a rat model [88,89]. Animals were ovariectomized and treated with E2-CDS prior to MCAO. Mortality was reduced from 65% in vehicle treated animals to 16% in rats receiving E2-CDS, a finding that paralleled a reduction in ischemic area from 26% in vehicle-dosed animals to 9% in E2-CDS treated rats. These data as well as the activity demonstrated by E2, provided the first evidence that estrogens exert neuroprotective effects in animals models of ischemia.

6. Route of administration

Initial experiment protocols were carried out using the i.v. administration route. While there may be some specialized utility for this approach, more convenient formulations would appear to be necessary in the successful development of an E2-CDS product. Several suggestions were considered. Preliminary pharmacological studies have suggested that both buccal (Figure 19) and transdermal application (Figure 20) leads to potent estrogenic activity and have, therefore, been selected for further exploration. Buccal studies have been completed through the Phase I clinical stage while transdermal studies are still at a preclinical/feasibility stage.

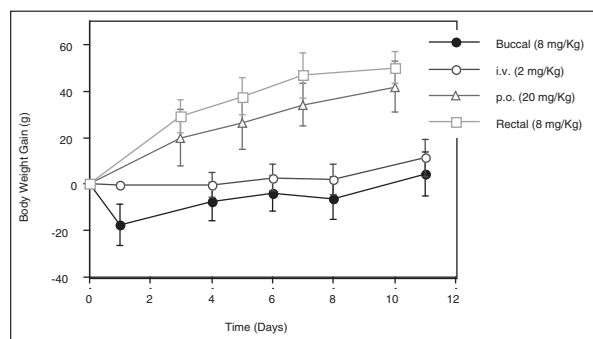


Figure 19 - Effect of various administration routes of E2-CDS solubilized in HP β CD on weight gain in rats.

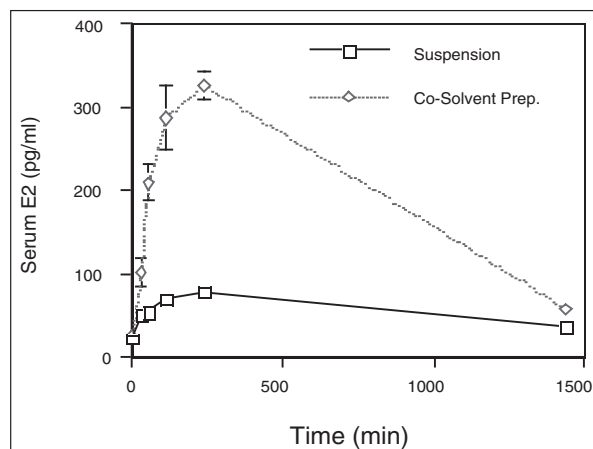


Figure 20 - Transdermal uptake of E2-CDS from various ointment formulations.

7. Toxicology

Extensive safety evaluations were conducted on the E2-CDS. The E2-CDS formulated in 2-hydroxypropyl- β -cyclodextrin was shown to lack mutagenic potential in bacteria as measured in a standard Ames test [31]. The cyclodextrin was chosen as an excipient since it is not parenterally toxic and gives adequate aqueous solubility for the E2-CDS [90,91]. The E2-CDS system was tested in Sprague-Dawley rats and cynomolgus monkeys over a period of either 14 (subacute) or 90 (subchronic) days. In subacute studies, groups of 10 rats (5 males and 5 females) or 2 monkeys (1 male and 1 female) were injected i.v. with either normal saline, 2-hydroxypropyl- β -cyclodextrin in sterile water, or E2-CDS in the cyclodextrin vehicle at doses of 0.025, 0.25, 1.5 and 5.0 mg/kg. The treatments were administered every second day resulting in cumulative doses of 0.2, 2, 12, and 40 mg/kg. Twenty-four hours after the eighth dose, animals were sacrificed and necropsied. During the period of drug administration, animals were monitored for toxic manifestations and macroscopic observation of the animals was made at necropsy. Histopathological samples were prepared from various organs and blood was drawn for hematologic and clinical chemistry evaluation. No effects of the vehicle were apparent. The estrogen caused expected changes in hematology (increased neutrophils) in both species. In rats, various expected estrogenic reactions were observed, including weight loss and pituitary hypertrophy. In the subchronic paradigm, 20 rats (10 males and 10 females) and 8 monkeys (4 males and 4 females) were used. Again, one group received normal saline and, a second, the cyclodextrin vehicle at a dose of 200 mg/kg. E2-CDS was administered to monkeys at doses of 1.0 and 5.0 mg/kg and to rats at a dose of 2.5 mg/kg. Drug treatment was performed every other day, resulting in cumulative doses of 46 and 230 mg/kg in monkeys and 115 mg/kg in rats. Twenty-four hours after the 46th dose, animals were necropsied and biological samples obtained for evaluation. There was no mortality in this study as in the subacute trials and no abnormal cageside behaviors were observed. Again, various estrogen-sensitive parameters were significantly altered as a result of E2-CDS dosing, including decreased body weight and white blood cell count and increased pituitary weight in rats and decreased protein and glucose concentrations with increased triglycerides in monkeys. The toxicologic evaluation of the data obtained from the subchronic studies indicated that the no observable adverse effect dose was the maximum dose used in the protocol, i.e. 5 mg/kg in monkeys and 2.5 mg/kg in rats (Table V).

Table V - E2-CDS toxicology summary.

- Ames test (salmonella reverse mutation assay): negative
- Range finding (2 weeks, i.v., rats and monkeys)
No unexpected findings (5.0 mg/kg/2d)
No alteration in neurochemistry
No alteration in neurohistology
- Subchronic (13 weeks, i.v., rats and monkeys)
No unexpected findings (5.0 mg/kg/2d in monkeys and 2.5 mg/kg/2d in rats)

As the CDS, by its design, targets drugs to the CNS, the neurotoxicologic potency of these compounds was examined. This was done for E2-CDS by evaluating the effect of the CDS

on central neurochemical function [92]. In the experiment, E2-CDS, saline, or the cyclodextrin vehicle was administered to conscious, restrained cynomolgus monkeys i.v. every other day for 2 weeks. The cumulative dose of E2-CDS ranged from 0.2 to 40 mg/kg and two monkeys (one male and one female) were included in each group. Twenty-four hours after the eighth dose, animals were euthanized and samples of striatum removed and frozen. During the study, animals were observed twice a day for locomotor impairment such as rigidity, akinesia, and hypoactivity. No altered motor activity was observed. Neurochemical analysis of brain samples was performed by HPLC with electrochemical detection. The results indicated that there was no effect of E2-CDS on striatal dopaminergic concentration [92]. The unaffected dopamine levels and the lack of behavioral changes suggest neurotoxicologic safety of these materials.

8. Clinical investigation

The first human examination of E2-CDS was designed as a rising dose safety study and utilized HP β CD as the vehicle (Table VI) [93]. In this evaluation, menopausal women served as the test population in that plasma LH and FSH could be readily assayed as indicators of pharmacological action. The initial report evaluated eight subjects. In the protocol, all subjects were healthy, post-menopausal females as defined by age and time of last menstrual period (LMP), as confirmed by plasma FSH and LH levels. Each subject received a single dose of E2-CDS as an intravenous injection of the drug dissolved in 20% (w/v) HP β CD. Subjects remained for 48 h in the clinic and subjective side-effects and vital signs were recorded. Blood samples were obtained for analysis of LH, FSH and 17- β -estradiol at 15 and 30 min, 1, 2, 4, 8, 24 and 48 h after drug administration. Subjects were then released from the clinic but were asked to return on the morning of day 4 and day 7 for additional blood sampling. In addition, pre- and post-study blood samples were taken for clinical evaluation. In the study, all subjects met the entry criteria for inclusion. There were no dropouts and all subjects completed the protocol. No adverse effects were reported which could be attributed to the E2 and all hematological and clinical chemistry values were unaffected by drug administration. The results appeared to cluster into three groups: 10-40 μ g (n = 3), which showed minimal changes; 80-640 μ g, which showed threshold effects (n = 5); and 1280 μ g (n = 2) which showed substantial and sustained decreases in plasma LH levels. Grouping the data for interpretation also has the benefit of reducing the effects of inter-individual variation as only one subject was given a particular dose of drug in most cases.

Figure 21 illustrates the relationship between these three dose groups and the percent maximum fall in LH value [93].

Table VI - E2-CDS clinical protocol for the first human clinical trial.

- Conducted by Simbec Ltd., Wales, UK
- Post-menopausal volunteers (n = 8, LH > 128 IU/ml, serum E2 < 68 pg/ml)
- Single rising dose, open label, tolerance study (i.v. in 20% HP β CD, 1 to 1280 μ g and 900 μ g E2)
- Clinical chemistry, hematology, urinalysis, EKG, etc.
- LH and E2 levels (0 to 168 h)

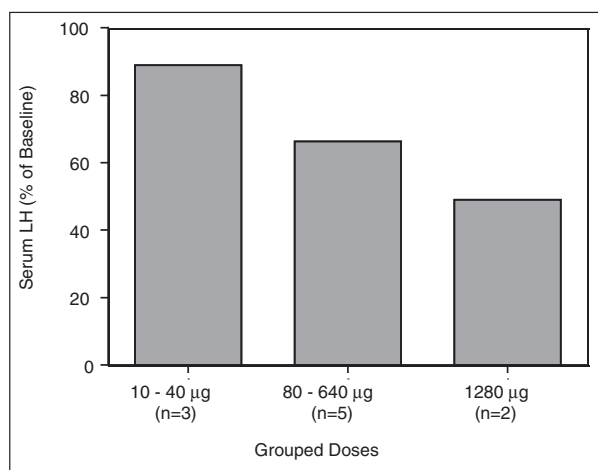


Figure 21 - Effect of E2-CDS on LH levels in female volunteers.

Mean peak decreases of 11, 34 and 50% for the three groups were noted. This fall in LH favorably compares to mean peak decreases of 28-36% observed after 1 month of dosing with E2 transdermal patches [94, 95]. In these studies, it was difficult to quantitate the magnitude of improved action obtained using the CDS relative to the natural hormone E2 in that no studies have been published which specifically examine the effect of moderate i.v. dose of E2 on serum LH and E2. This lack of experimental data was not totally unexpected since E2 is poorly water-soluble. This makes parenteral dosing especially difficult when moderate to high E2 doses are considered. The recent development of non-toxic, highly water-soluble, modified β -cyclodextrin has allowed for the i.v. administration of highly water-insoluble compounds such as sex steroids. The paucity of historic controls and the timely development of safe, useful and water-soluble inclusion complexes of E2 led us to conduct an i.v. study using this technology to examine E2 and LH levels with time after moderate doses of E2. *Figure 22* illustrates the effect of E2 and E2-CDS, both administered in an aqueous-modified β -cyclodextrin vehicle, on LH levels at various times post-treatment in a representative post-menopausal human volunteer. In this study, the dose of E2 was 900 µg and the equimolar dose of E2-CDS was 1280 µg. The data were reported as percent of control where control values were obtained by averaging the LH levels obtained at time 0 and on the day prior to treatment. As shown, LH is dampened in a clinically meaningful way through 96 h after E2-CDS treatment, while LH values have already returned to control values by 24 h after E2 dosing [31]. The LH suppression induced by E2-CDS returns to control values by 168 h. As would be expected for a long-acting drug such as E2-CDS, the ratio of the areas under the LH suppression curves obtained after either E2-CDS or E2 dosing, increased as a function of time. In addition, if the initial LH drop which is common to both treatments is discounted, the apparent E2-CDS/E2 enhancement is even greater with the ratio of E2-CDS to E2 (examining the AUC from 24 to 168 h) being greater than ten-fold. The effect of E2 or E2-CDS on serum E2 is given in *Figure 23*. Administration of E2 in an HP β CD vehicle rapidly generates high serum E2 levels as expected and these levels rapidly fall. In contrast, E2-CDS produces relatively low initial

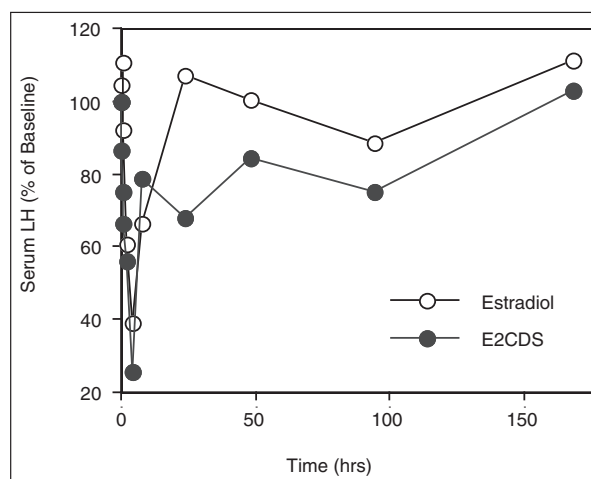


Figure 22 - Effect of E2-CDS or E2 on LH in a female volunteer.

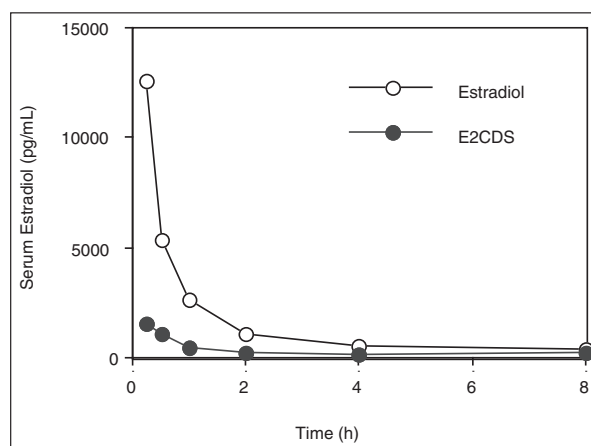


Figure 23 - Effect of E2 or E2-CDS on serum E2 in a female volunteer.

levels of E2. These concentrations are also rapidly cleared. After 8 h, the profiles of E2 elimination are superimposed subsequent to either E2 or E2-CDS administration.

Two important pieces of information are derived from this study: i) The initial levels of plasma E2 generated by E2-CDS are five- to ten-fold less than those generated after E2. This dampened surge should improve the therapeutic index of E2-CDS relative to E2, especially in circumstances where estrogens are contraindicated such as in breast cancer. The levels of E2 following E2-CDS treatment do, however, seem to be sufficient to support bone development in post-menopausal women. ii) LH is clearly suppressed at 24, 48 and 96 h in a clinically relevant manner after E2-CDS treatment, while no LH suppression is evident at 24 h and beyond after E2 treatment. This occurs in spite of the fact that the serum levels of immunoreactive E2 are similar for the two groups. This strongly suggests that E2-CDS is indeed working via a brain-mediated mechanism in that serum differences in E2 cannot account for the LH suppression.

A second clinical evaluation was subsequently performed (*Table VII*) [96]. The purpose of this second trial was to: i) increase the number of patients exposed to the E2-CDS, ii) collect further safety information on the E2-CDS in humans,

Table VII - E2-CDS clinical protocol for the second human clinical trial.

- Conducted by Pharmakopius, Goring on Thames, UK
- Post-menopausal volunteers (n = 18) on their initial visit received 1.0 mg of Progynova (E2 valerate) and were subsequently randomized into two subgroups
- Single rising dose, open label, tolerance study (i.v. in 20% HP β CD (0.6 to 2.5 mg) or buccal in an reconstituted HP β CD solution (2 to 16 mg))
- Subsequent to the E2-CDS treatments, 9 women were treated with E2 in HP β CD equimolar to the 2.5 mg dose of E2-CDS
- Clinical chemistry, hematology, urinalysis, etc.
- LH, FSH and E2 levels

iii) to establish the feasibility for buccal dosing in humans and iv) to demonstrate the central action of the E2-CDS relative to a both a commercially available oral preparation as well as parenteral estradiol. The study designed included 18 woman who received an oral preparation of E2 valerate (Progynova) on their first visit followed by clinical and endocrinological follow-up. The group was then randomized into two subgroups that were to receive either E2-CDS complexed with HP β CD buccally or i.v. The doses in both arms of the study were administered in a rising fashion starting at 0.6 mg in the i.v. portion of the study and 2.0 mg in the buccal arm. The highest doses examined were 2.5 mg i.v. and 16 mg buccally. At the end of the experiment, the groups were recombined and nine of the eighteen woman were selected for an i.v. dose of E2 (solubilized in HP β CD equimolar to the 2.5 mg E2-CDS dose). For all treatments, clinical chemistry and hormone levels were determined. Both i.v. and buccal dosing demonstrated a dose-related suppression of circulating LH levels (*Figure 24*) [96]. A comparison of buccal doses with biological effect suggested significant bioavailability. A comparison of the area under the LH suppression curves and plasma E2 curves for E2-CDS and the commercially available oral preparation suggested that the E2-CDS was significantly more active (as measured by LH suppression) for a certain blood level of E2 than was the Progynova. This was manifested after either buccal or i.v. administration (*Figure 25*). In a comparison of i.v. E2 *versus* i.v. E2-CDS, similar results to the initial trial were documented. Thus, while E2 and E2-CDS exerted similar effects on LH suppression, the E2-CDS elevated E2 levels only a small fraction (4%) as compared to i.v. E2 administration (*Figure 26*). The results of this limited study indicated that the E2-CDS is acutely safe at levels far in excess of those contemplated for commercial use. In addition, the evaluation found that E2-CDS was bioavailable buccally and that, if anything, the doses administered overestimated the potential clinical need. A cursory pharmacokinetic evaluation of the data also suggested that peripheral estradiol levels can be easily manipulated through alterations in dosing amounts and frequency. This is related to the long half-life of E2-CDS in the CNS. Thus a dosing schedule can be devised that minimizes plasma E2 as a function of central E2 or that produces a particular peripheral level of E2.

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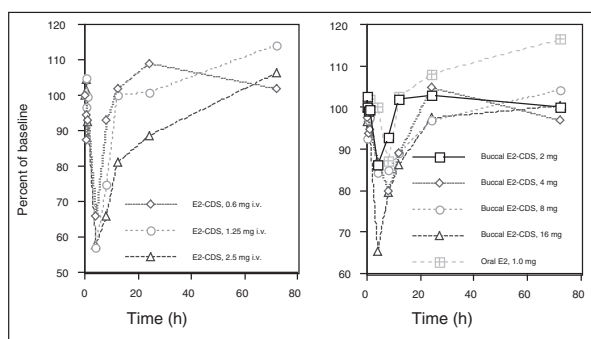


Figure 24 - Effect of E2-CDS on LH levels in post-menopausal volunteers.

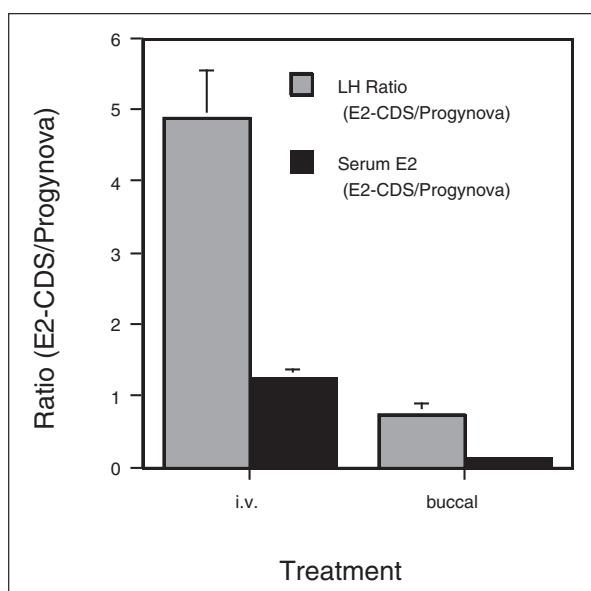


Figure 25 - Effect of E2-CDS or Progynova on LH and serum E2 ratios.

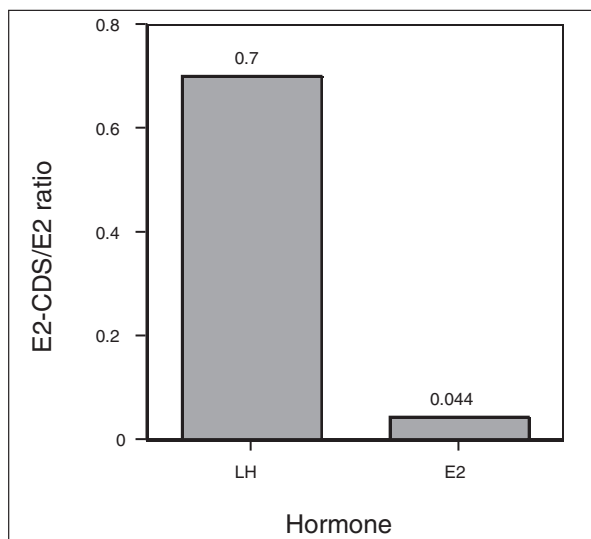


Figure 26 - Effect of parenteral E2 or E2-CDS on LH and serum E2.

E2-CDS has been demonstrated to be a useful, potent and long-lasting estradiol delivery system that targets the brain and central nervous system. The use of HP β CD has allowed this material to be conveniently formulated and administered and indeed the product would not have been developable without this functional excipient. Cyclodextrins have provided formulations intended for buccal/sublingual, intravenous, transdermal or vaginal use. Clinical evaluations of the E2-CDS suggest a potent central effect with only marginal tendencies to elevate systemic estrogen levels. Importantly, peripheral levels appear to be easily manipulated using the delivery system as a function of dosing amount and frequency. Taken as a whole, the data suggest that the E2-CDS may be a useful and safe therapy for menopausal symptoms as well as estrogen-dependent cognitive deficits, including Alzheimer's disease.

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MANUSCRIPT

Received 17 November 2003, accepted for publication 25 November 2003.