Development of 17α-Estradiol as a Neuroprotective Therapeutic Agent

Rationale and Results from a Phase I Clinical Study

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ABSTRACT: 17α-estradiol (17α-E2) differs from its isomer, the potent feminizing hormone 17β-estradiol (17β-E2), only in the stereochemistry at one carbon, but this is sufficient to render it at least 200-fold less active as a transactivating hormone. Despite its meager hormonal activity, 17α-E2 is as potent as 17β-E2 in protecting a wide variety of cell types, including primary neurons, from a diverse array of lethal and etiologically relevant stressors, including amyloid toxicity, serum withdrawal, oxidative stress, excitotoxicity, and mitochondrial inhibition, among others. Moreover, both estradiol isomers have shown efficacy in animal models of stroke, Alzheimer's disease (AD), and Parkinson's disease (PD). Data from many labs have yielded a mechanistic model in which 17α-E2 intercalates into cell membranes, where it terminates lipid peroxidation chain reactions, thereby preserving membrane integrity, and where it in turn is redox cycled by glutathione or by NADPH through enzymatic coupling. Maintaining membrane integrity is critical to mitochondrial function, where loss of impermeability of the inner membrane initiates both necrotic and apoptotic pathways. Thus, by serving as a mitoprotectant, 17α-E2 forestalls cell death and could correspondingly provide therapeutic benefit in a host of degenerative diseases, including AD, PD, Friedreich's ataxia, and amyotrophic lateral sclerosis, while at the same time circumventing the common adverse effects elicited by more hormonally active analogues. Positive safety and pharmacokinetic data from a successful phase I clinical study with oral 17α-E2 (sodium sulfate conjugate) are presented here, and several options for its future clinical assessment are discussed.

KEYWORDS: Alzheimer's disease; Parkinson's disease; amyotrophic lateral sclerosis; Friedreich's ataxia; mitochondria; apoptosis; lipid peroxidation

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INTRODUCTION

Several open-label trials have indicated that hormone replacement therapy (HRT) provides cognitive benefit to Alzheimer's disease (AD) patients,[1–9] as have several prospective, placebo-controlled, double-blind studies.[10,11] Moreover, several epidemiological studies have indicated that HRT could postpone the onset and severity of AD.[12,13] Conversely, other studies have failed to show benefit of estrogens in women with mild to moderate AD.[14–16] Several large trials have recently shown that initiating HRT, with either conjugated equine estrogens (CEEs) alone or CEEs opposed by medroxyprogesterone, in women more than 65 years old neither provides cognitive benefit nor forestalls the onset of dementia.[17–20]

The above studies were conducted with CEE, a complex mixture containing more than 100 compounds.[21] As a result, assessing the contribution of individual components of CEEs to either positive or negative effects is impossible unless each component is independently evaluated. Indeed, several studies using transdermal 17β-estradiol (17β-E2) instead of CEEs found improved performance in attention, verbal memory, visual memory, and semantic memory in postmenopausal women with AD.[22–24]

Crucial to the clinical development program described here, many *in vitro* and *in vivo* studies indicate that 17α-estradiol (17α-E2), a stereoisomer of 17β-E2 that is at least 200-fold less active as a hormone,[25] protects cells and animals against a variety of toxic stressors germane to neurodegenerative diseases as well as the potently feminizing hormone 17β-E2. This fundamental dichotomy provides a way to separate the hormonal effects of these molecules from their cytoprotective activities and has correspondingly fostered the development of 17α-E2 as a potential neuroprotective treatment for neurodegenerative diseases, including AD and Parkinson's disease (PD), among others. Such broad-spectrum utility is possible because the mechanistic data indicate that 17α-E2 and other analogues stabilize mitochondrial function under cytotoxic conditions, including excessive Ca^{2+} mobilization and free radical exposure. By repressing mitochondrial collapse, these molecules target fundamental pathways of cell death regardless of initial insult, and they correspondingly forestall both apoptosis and necrosis under a wide range of pathogenic circumstances.

This minireview summarizes some of the studies demonstrating *in vitro* and *in vivo* cytoprotection by 17α-E2 and in so doing outlines the rationale underlying its clinical development for degenerative diseases. The current mechanistic models responsible for cytoprotection are also presented, as are safety and pharmacokinetic data from a phase I clinical trial undertaken in anticipation of further clinical development.

CYTOPROTECTION IN CELL-BASED STUDIES

The key observation that has fostered the clinical development of 17α-E2 is that it has neuroprotective efficacy comparable to that of 17β-E2 in SK-N-SH human neuroblastoma cell lines exposed to serum deprivation and hypoglycemia.[26–28] SK-N-SH apoptosis is not moderated by cholesterol, testosterone, dihydrotestosterone, progesterone, or corticosterone, and a 100-fold molar excess of tamoxifen antagonizes only one-third of the neuroprotective effects of either estradiol isomer, indicating that most of the neuroprotection is not mediated via a tamoxifen-antagonized receptor mechanism.[26,29] These SK-N-SH neuroblastoma cells, and the closely related SH-SY5Y cells used in many of the studies described below, are dopaminergic in origin, so that demonstrating cytoprotection in these cells is particularly germane for the neuronal loss characteristic of PD.

The cytoprotective effects for both 17α-E2 and 17β-E2 are also reported from many other cell models, including excitotoxicity where cell death is due to mitochondrial failure and ensuing oxidative stress.[30–32] For example, 17α-E2 and 17β-E2 are equipotent in their ability to protect primary rat hippocampal neurons from gp120 toxicity, a pathology precipitated by a combination of *N*-methyl-D-aspartate (NMDA) receptor activation, accumulation of intracellular calcium, generation of reactive oxygen species (ROS), and ensuing lipid peroxidation.[33] As above, tamoxifen does not reduce the protection afforded by either 17β-E2 or 17α-E2, suggesting that the mechanism is independent of classical estrogen receptors ERα and ERβ.[33] Similarly, 17β-E2, 17α-E2, and estrone all attenuate oxidative neuronal death induced by excitotoxicity, oxidative stress, and serum deprivation–induced apoptosis in primary mouse cortical neurons.[34] This neuroprotection is also not blocked by the specific estrogen receptor antagonist ICI 182,780, nor does addition of the antioxidant Trolox increase protection, indicating that direct effects by all three molecules are responsible for cytoprotection. Comparable protection by both 17β-E2 and 17α-E2 against glutamate- and nitric oxide (NO)–induced motor neuronal death has also been reported in primary cultures of rat spinal cord.[35] Such data encourage the notion that nonfeminizing estradiol analogues may have utility as a treatment for amyotrophic lateral sclerosis (ALS) in which motor neuron loss is the cause of pathology, and where both mutations in superoxide dismutase (SOD) and mitochondrial instability contribute to the disease. Finally, the cytotoxic effect of oxidized low-density lipoprotein on PC-12 neuronal cells in culture is also moderated in a dose-dependent manner by several estrogen analogues, including 17α-E2, which—although less potent than others—still provides almost 50% protection.[36]

In cell models germane to PD, both 17α-E2 and17β-E2, but not corticosterone, testosterone, or cholesterol, protect dopaminergic neurons from cytotoxic oxidative stress induced by glutamate, superoxide anions, or hydrogen peroxide.[37] Neuroprotection is not blocked by either an estrogen receptor an-

tagonist or a protein synthesis inhibitor, indicating that neuroprotection against glutamate is mediated neither by classical estrogen receptors nor by activation of genome transcription. Later studies of nigral neuronal apoptosis, induced by exposure to either bleomycin sulfate or buthionine sulfoximine, suggest that the antiapoptotic neuroprotection afforded by 17α-E2 and 17β-E2 is mediated by transcription through an activator protein 1 (AP-1) site downstream from JNK and caspase-3 activation.[38]

Regardless of pathway, both 17α-E2 and 17β-E2 protect dopaminergic neurons in culture from cell death induced by methylphenylpyridinium (MPP^+).[39] Similarly, both 17β-E2 and 17α-E2 protect dopaminergic and nondopaminergic neurons from toxicity induced by 6-hydroxydopamine and by high MPP^+ concentrations (50 μM), but not by lower MPP^+ concentrations (<10 μM), which are usually associated with mitochondrial inhibition.[40] These observations suggest that neuroprotection by 17α-E2 or 17β-E2 might be due to their antioxidant properties, although other nongenomic effects should not be excluded.[40] Such observations are corroborated by data showing that 17β-E2, 17α-E2, and the phytoestrogens quercetin and resveratrol all protect pheochromocytoma PC12 cells against MPP^+, whereas closely related molecules with less extended resonance structure, such as coumestrol, genistein, and kaempferol, did not (see discussion in MECHANISM-OF-ACTION MODELS section below).[41]

In cell models more germane to AD, the neurotoxic fragment of β-amyloid (Aβ 25-35) causes a dose-dependent death of SK-N-SH cells that is significantly attenuated by both 2 nM 17α-E2 and 17β-E2,[42] supporting the hypothesis that cytoprotection is independent of hormonal potency. Similarly, in mature hippocampal neurons, both 17α-E2 and 17β-E2 partially prevent neuronal death induced by fibrillar Aβ, an effect correlated with the formation of a more dynamic microtubular system.[43] Equal potency for both 17α-E2 and 17β-E2 against β-amyloid peptide–induced toxicity is also seen in PC12, neuroblastoma, and T47D human breast cancer cells.[44] However, not all studies find equal potency of the two isomers. For example, the extensive death of SN56 murine cholinergic cells induced by 1–40 fragment of amyloid-β peptide is moderated in a dose-dependent manner by 17β-E2 through a mechanism that is blocked by ICI 182,780, indicating involvement of classical estrogen receptor activation.[45] In this study, the "inactive isomer" 17α-E2 consistently showed weaker neuroprotection than the native hormone, but this response was unaffected by ICI 182,780. Indeed, when several indicators of gene expression were used, 17β-E2 elicited receptor-mediated hormonal responses in this system, whereas 17α-E2 did not.[45] These data suggest that 17β-E2 is neuroprotective against β-amyloid–induced toxicity by activation of classical estrogen receptor–mediated pathways, plus other effects, whereas the less potent cytoprotection shown by 17α-E2 arises primarily via alternative, nonclassical receptor or receptor-independent, pathways. This conclusion is supported by similar studies where 17β-E2 attenuates death of PC12

cells caused by the carboxy-terminal fragment of amyloid precursor protein, whereas 17α-E2 does not.[46] In this study, tamoxifen inhibited cytoprotection, suggesting that it is predominantly mediated by ER, and as such would be less amenable to moderation by 17α-E2 with its substantially less effective ER activation.

Interpretation of cell-based studies is confounded by differing susceptibilities of different cells to different stressors. For example, studies of PC12 and Neuro2a neuroblastoma cells show that PC12 cells are 200-fold more susceptible than Neuro2a cells to toxicity caused by exposure to glutamate, H_2O_2, acetylcholinesterase (AChE)–amyloid-β peptide (Aβ) complexes, or to Aβ 25-35.[47] Conversely, both cell lines are equally susceptible to nonoxidative cytotoxic insults, such as those caused by Triton X-100 and serum deprivation. Interestingly, both 17α-E2 and 17β-E2 are equally protective of PC12 and Neuro2a cells, perhaps because PC12 cells maintain five-fold less glutathione than the Neuro2a cells, which would serve to exacerbate oxidative toxicity in the latter.[47] This may be particularly germane given the mechanistic model presented below.

MECHANISM-OF-ACTION MODELS

Regardless of the mechanistic pathways responsible for cytoprotective activities of 17β-E2, the evidence is compelling that 17α-E2, and other nonhormonal analogues, operate via mechanisms independent of classical ERs. These include an array of nonclassical estrogen-binding proteins in the rodent brain, as well as extraneural targets of estrogen.[48] This topic is no doubt covered elsewhere in this volume, but it should be noted that in the developing rodent cerebral cortex, 17α-E2 elicits a rapid and sustained activation of multiple signaling proteins within the mitogen-activated protein (MAP) kinase cascade, including B-Raf and extracellular signal–regulated kinase (ERK).[49] These data support the hypothesis that a novel, estradiol-sensitive and ICI-182,780–insensitive estrogen receptor may mediate 17α-E2–induced activation of ERK in the rodent brain, and perhaps in the human brain as well.

Nevertheless, many studies of structure–activity relationships (SARs) have shown that the molecular motifs required for cytoprotection are significantly different from those required for ER-dependent gene transcription. For example, 17α-E2, and several other nonhormonal estradiol derivatives, prevent intracellular peroxide accumulation and, ultimately, the death of primary neurons, clonal hippocampal cells, and cells in organotypic hippocampal slices when exposed to cytotoxic insults including amyloid Aβ protein, glutamate, hydrogen peroxide, and buthionine sulfoximine.[50] Such protection is seen only with estradiol derivatives containing the hydroxyl group in the C-3 position on the A ring of the steroid molecule and is independent of activation of ERs.[50]

Similarly, only estrogen analogues containing an intact phenolic A ring provide cytoprotection against apoptosis induced in SK-N-SH cells via serum withdrawal.[28] In this study, A-ring substitutions, such as 3-*O*-methyl ethers, are inactive. Additional SAR studies have led to the conclusion that a phenolic A-ring, and at least two to three rings of the steroid nucleus, are necessary for neuroprotective activity.[51] Convincingly, 17α-E2 provides neuroprotection of the HT-22 murine hippocampal cells, a cell line lacking functional classical ERs, against neurotoxicity by β-amyloid peptide.[42] Importantly, neuroprotective efficacy of both 17α-E2 and 17β-E2 in HT-22 cells is increased by an average of 400-fold by the addition of exogenous glutathione, leading to the conclusions not only that a nuclear estrogen receptor is unnecessary for the neuroprotective actions of 17α-E2 but also that glutathione greatly amplifies cytoprotective efficiency.[29,51] An even larger glutathione effect is reported from studies of amyloid A toxicity.[52]

These steroids are lipophilic (17α-estradiol calculated log P [clogP] = 3.91), leading many to focus on cellular hydrophobic domains, such as membranes, as a likely site of action. Fourier transform infrared spectroscopy has shown that 17β-E2, tamoxifen, and to a lesser extent 17α-E2, decrease the fluidity of rat striatal and frontal cortex membranes, indicating direct, stereospecific, membrane effects independent of ERs.[53] Additional evidence of direct membrane effects of 17α-E2 and other estratrienes is found in studies on lipid packing that show decreased membrane fusion events and fluidity.[54]

The requirement for the phenolic A ring and extended steroid resonance for cytoprotective activity indicates that intrinsic free-radical scavenging contributes to the receptor-independent neuroprotective effects of estrogens.[55] Scavenging of the hydroxyl or perhydroxyl radical ($^{\bullet}$OH) by estradiol produces a quinol, a form that has no affinity for ERs, which is then reduced back to the original form via an enzyme-catalyzed reaction using NAD(P)H as a coenzyme and/or via direct reduction by glutathione.[55–57] Indeed, the quinol obtained from 17β-E2 oxidation attenuates glutamate-induced oxidative stress in the HT-22 cells that lack ERs, providing evidence of this redox cycle.[55]

MITOCHONDRIAL EFFECTS

The current mechanistic model holds that 17α-E2, and other polycyclic phenols with a planar geometry, intercalate into cell membranes, where they terminate lipid peroxidation chain reactions by redox cycling the steroidyl radical with glutathione and/or some other source of reducing potential.[56,57] For example, 17α-E2 and other cytoprotective steroids moderate lipid peroxidation in artificial micelles induced by a hydrophobic prooxidant. Moreover, the rate of hydroperoxide formation in this micelle model is repressed by addition of reduced glutathione, indicating direct, nonenzymatic redox cycling

with the steroids.[56] Additional studies with oxidatively stressed human red blood cells show accelerated hexose monophosphate shunt flux in the presence of cytoprotective steroids, thereby providing direct evidence of accelerated glutathione turnover in cells when treated with 17α-E2 and other cytoprotective, but not with noncytoprotective, analogues. These data indicate that 17α-E2 and other phenolic steroids serve as catalysts, allowing the reducing potential of the metabolically maintained glutathione pool to rectify membrane peroxidation.[56,57]

Such a membrane stabilization mechanism would be particularly germane to mitochondria, where mitochondrial integrity, and hence cellular viability, is determined by the impermeability of the inner membrane and consequent retention of the mitochondrial membrane potential ($\Delta\Psi m$).[37,56,57] In these studies, 17α-E2 (1 μM) effectively stabilizes mitochondrial integrity against pathogenic Ca^{2+} loads sufficient to induce mitochondrial collapse in untreated mitochondria. Noncytoprotective estradiol analogues lacking the A-ring phenol do not protect mitochondria from Ca^{2+} overload. Importantly, glutathione pools within the mitochondria are actively maintained at extraordinarily high concentrations, typically 8–10 mM, which would account for the high potency of 17α-E2 and analogues at the level of mitochondrial function *in vivo*.[57]

Direct mitoprotective effects of 17α-E2 on respiration and coupling characteristics have also been demonstrated using purified rat brain mitochondria submitted to anoxia reoxygenation.[58] After anoxia reoxygenation, 17α-E2 (1 μM) significantly reverses uncoupling by restoring state 4 respiration, a reflection of membrane integrity, and 17α-E2 also preserves mitochondrial membrane potential under oxidative stress via nongenomic effects.[58,59]

Mitochondrial stabilization by 17α-E2 and other estradiols should have several observable, physiological consequences, and, in fact, 17α-E2 significantly preserves mitochondrial membrane potential, intracellular ATP levels, and cell viability in cultured human lens epithelial cells after exposure to H_2O_2.[60]

OTHER MECHANISMS

In addition to membrane stabilization and retention of mitochondrial function, 17α-E2 and its phenolic analogues could have other activities that also contribute to cytoprotection. For example, 17α-E2, 17α-ethinylestradiol, the scavestrogens J811 and J861 (estrogen analogues developed by augmenting *in vitro* antioxidant activity), and 17α-E2 moderate potentially mitotoxic and hence cytotoxic Ca^{2+} influx in human aortic smooth-muscle cells.[61] Comparable moderation of pathogenic Ca^{2+} mobilization has been reported in immortalized hypothalamic neurons (GT1-7 cells) after exposure to the cytotoxic fragment of amyloid protein, Aβ 25-35.[62] Preadministration of

17β-E2, 17α-E2, phloretin, and cholesterol, which influence membrane fluidity, significantly decreased the rise in intracellular Ca^{2+} levels. These findings support the idea that disruption of Ca^{2+} homeostasis by Aβ 25-35 may be an important molecular basis of its neurotoxicity and that membrane properties may play key roles in neurotoxicity by amyloid.

Other mechanisms of action of potential importance to cytoprotection include allosteric modulation of key metabolic regulatory enzymes. For example, the oligomycin sensitivity-conferring protein subunit of the mitochondrial F_0F_1–ATP synthase has an estrogen-binding domain through which estrogen and its analogues can directly modulate cellular energetic metabolism.[63] Both 17α-E2 and 17β-E2 effectively bind to the oligomycin sensitivity–conferring protein, leading to the notion that E2 and its analogues may interact with ATP synthase/ATPase, thereby modulating cellular energy metabolism.[64] It should be noted in this context that adenylates are among the most potent inhibitors of the mitochondrial membrane permeability transition, the process responsible for irreversible mitochondrial collapse that induces apoptosis and necrosis.[30–32] By preserving adenylate charge during hypoxia or excessive Ca^{2+} loading, estrogens may also be forestalling mitochondrial collapse and cell death.[56,57]

IN VIVO EFFICACY

Using a transgenic animal model of AD, Levin-Allerhand *et al.*[65] found that both 17α-E2 and 17β-E2 significantly repress amyloid levels. In this study, Swedish mutant Aβ precursor protein transgenic mice were ovariectomized or sham ovariectomized at 4 weeks of age and treated with placebo or 17β-E2 or 17α-E2 as a slow-release subcutaneous (s.c.) implant. Although total Aβ levels were not different between sham- and actually ovariectomized mice, levels of Aβ were decreased by both 17β-E2 or 17α-E2 treatments. These authors concluded that better efficacy of 17α-E2 compared with 17β-E2, and the paucity of hormonal effects, should encourage its use for AD.

Better efficacy of 17α-E2 has been found using a rat model of AD where cholinergic function in cortex and hippocampus is impaired via intracerebroventricular administration of the neurotoxin 1-ethyl-1-(2-hydroxyethyl)-ethylenimine (AF64A).[66] The resulting oxidative degeneration of cholinergic neurons results in impaired cognitive function. In this study, only 52% of AF64A-treated animals correctly responded in an active avoidance task compared with 91% of the untreated control subjects. Treatment of AF64A animals with 17α-E2 (s.c. once daily, at 4 and 40 g/kg of body weight), improved the response, with fully 76% of the animals responding correctly. 17β-E2 was less effective in that this benefit was seen only at the highest dose of 40 g/kg, compared with 4 g/kg for 17α-E2.[66]

In an acute model of neuronal necrosis and apoptosis, both 17β-E2 and 17α-E2 potently reduce infarct volume within the cerebral cortex following occlusion of the middle cerebral artery.[67,68] Moreover, the enantiomer of 17β-E2, as well as 2-adamantylestrone and 17-desoxyestradiol, are as effective as 17β-E2 and 17α-E2 in reducing lesion volume. Similar data have also been reported from a model of cerebral ischemia in ovariectomized gerbils subjected to transient global ischemia.[69]

The effects of 17α-E2 on visual (object recognition) and place (object placement) memory have been investigated in ovariectomized rats.[70] Treatment with 17α-E2 (single s.c. injection, 15 g/kg) significantly improves scores for the time spent exploring both new objects, and for recognizing objects at a new location, in recognition and retention trials compared with control animals. 17α-E2 showed a similar effect in the object recognition test but required a higher dose of 60 g/kg to show a comparable effect in the object placement tests.[70]

Although both 17α-E2 and 17β-E2 show comparable cytoprotective activity against MPTP toxicity in various cell culture stsyems,[38–41] only 17β-E2 has consistently shown efficacy in the *in vivo* models of PD using MPTP as the lesioning agent.[71–74] This discrepancy may be due to sex issues, with female animals showing MPTP resistance or milder lesions and better response to 17β-E2,[73] as well to dosing issues, with physiological hormone levels eliciting better cytoprotection than pharmacological levels.[74] Nevertheless, the observation that cytoprotection *in vivo* occurs in the absence of ERs[75] suggests not only that stereospecificity may be less of an issue than experimental protocols but also that nonclassical ERs may be involved in these rodent models.

CLINICAL DEVELOPMENT OF 17α-E2

17α-E2 has generally been considered a xenobiotic in humans, primarily because endogenous levels are quite low. For example, in the phase I data presented below, initial 17α-E2 plasma levels were below the limit of quantification (<25 pg/mL) in all of the subjects. Moreover, earlier studies failed to detect 17α-E2 in urine of pregnant women.[76,77] However, a more sensitive urine assay later showed that 17α-E2 was consistently present on different days of the menstrual cycle in the urine of a normal woman (mean of 0.08 g/24 h, range 0.07–0.09 g/24 h)[78] and in the urine of 25 physiologically or surgically menopausal women.[79] It bears reiteration that interspecies differences can be profound; on a molar basis, the concentration of 17α–E2 is five-fold higher than 17β-E2 in urine from pregnant mares and in the resulting CEE preparations used for HT.[19]

In some species, interconversion between the stereoisomers can occur. For example, tritiated 17α-E2 was found in urine after intravenous administration

TABLE 1. Summary of pharmacokinetic parameters after single rising oral doses of 17α-estradiol sodium sulfate given to postmenopausal women[a]

Dose Level	Parameter	C_{max} (pg/mL)	T_{max}[b]	AUC 0 – t (pg/mL • h)	AUC 0 – infinity (pg/mL• h)	Lambda z (h–1)	$T_{1/2}$ (h)
50 µg	Mean	154.5	2.07	869.85	3312.19	0.1118	19.254
	SD	94.46	0.5-4.02	949.56	3016.88	0.0964	25.2161
100 µg	Mean	258.17	1.5	1937.58	3612.21	0.0502	17.177
	SD	186.95	1.0-4.0	1145.58	1534.2	0.0257	8.6402
200 µg	Mean	619.33	4	4707.62	7005.53	0.0467	16.5389
	SD	444.94	1.0-4.05	2469.97	3554.23	0.0207	4.7754

[a]Drug levels are total (free 17α-E2 + conjugated material after enzymatic cleavage).
[b]Median values and range are preseented for T_{max}.

of tritiated 17α-E2 in the male mongrel dog.[80] Similarly, s.c. injection of two female rabbits with estrone (60–100 mg per rabbit) yielded approximately 2–3 mg of 17α-E2 in the urine over 2–3 days.[81] Conversely, when rats were treated with 17α-E2, a conversion into the more estrogenically active 17β-E2 occurred.[80] However, aside from one report,[82] no such conversion has been observed in humans.[83–85] Taken together, these studies indicate that 17α-E2 is an endogenous estrogen in humans, albeit at low concentrations and dependent on the subject's sex and/or physiological status.

In addition to the phase I trial described below, there are several published studies of the administration of 17α-E2 to humans by oral, sublingual, s.c., intravenous, or topical routes.[82–90] These include studies with single oral doses ranging up to 70 mg, and a 12-week study at 2 mg p.o. per day. No adverse events have been reported.[82–90]

PHASE I STUDY

Given the paucity of clinical data with 17α-E2 as a single agent, single, rising oral doses of 17α-E2 (as the 3-sodium sulfate; MX-4509) were evaluated in eight healthy postmenopausal women, using a double-blind, placebo-controlled design. The objectives were to determine the safety (primary), tolerability (primary), and pharmacokinetics (secondary) of MX-4509 after single oral dose administration.

The subjects had a mean age of 60.4 years (SD 5.8), and all had prestudy serum estradiol levels less than 25 pg/mL and follicle-stimulating hormone levels greater than 20 IU/L. Subjects received each of the following treatments sequentially, according to a randomization code: study period 1, 50 g

oral 17α-E2 ($n = 6$) or placebo ($n = 2$); study period 2, 100 g oral 17α-E2 ($n = 6$) or placebo ($n = 2$); study period 3, 200 g oral 17α-E2 ($n = 6$) or placebo ($n = 2$). There were at least 7 days between dose administrations, and each study period was 24 h in duration. Subjects were given 17α-E2 as an oral solution containing either 50 g, 100 g, or 200 g of 17α-E2, dissolved in 50 mL of purified water.

Pre-study assessments were carried out during the 14-day period before the first dose and poststudy assessments took place 3–7 days after the final study procedure. Assessments included a physical examination, weight, blood pressure, pulse, respiration rate, 12-lead electrocardiogram assessments, laboratory safety screen, and follow-up of unresolved adverse events. Laboratory safety tests were performed pre-study, pre-dose, 24 h post dose and post study and included the following: (1) serum biochemistry: total protein, albumin, total bilirubin, glucose, sodium, potassium, bicarbonate, urea, creatine plus activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyle transferase (GGT); (2) hematology: red blood cell count, hemoglobin, hematocrit, mean cell hemoglobin, mean cell hemoglobin concentration, mean cell volume, white blood cell count and differentiation, platelet count; (3) coagulation: prothrombin time, partial thromboplastin time; (4) urinalysis: protein, glucose, specific gravity, ketones, urobilinogen, bilirubin, pH, blood.

All biochemistry, hematology, and urinalysis results were within reference ranges or, where they deviated, were not considered to be clinically significant by the investigating physician. All vital signs and electrocardiogram measurements were within the reference range or, where they deviated, were not considered to be clinically significant by the investigating physician. No clinically significant mean changes from baseline vital sign measurements were observed during the study. All three doses of 17α-E2 were well tolerated, no adverse events related to study medication were reported, and all subjects completed the study.

Plasma samples were collected over a 24-h period and were subjected to enzymatic cleavage to liberate 17α-E2 from its various conjugations. This was followed by extraction of total 17α-E2 present in a sample (unconjugated plus previously conjugated). The total 17α-E2 concentration was then determined using liquid chromatography–tandem mass spectrometry (LC–MS–MS).

Administration of 50, 100, and 200 g of 17α-E2 yielded peak plasma concentrations that were proportional with respect to C_{max} but were not proportional with respect to the overall extent of exposure (area under the curve; AUC). The median T_{max} ranged from 2.07–4.00 hours, but there was no statistically significant difference for T_{max} between the dose levels ($P = 0.61$). The elimination half-life was dose dependent and varied from 16.5–19.2 hours (FIG. 1) Levels of 17α-E2 in plasma were also measured, but no formal pharmacokinetic analysis was performed since subtraction of baseline levels of

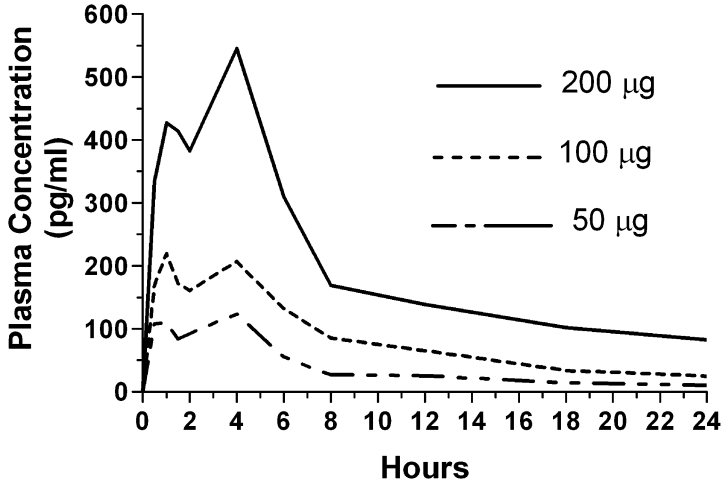

FIGURE 1. Plasma levels of 17α-E2 in escalating dose phase 1 trial. Healthy postmenopausal women were provided with the indicated doses of the sodium sulfate form of 17α-E2 in water, and plasma concentrations were assessed by a LC–MS–MS assay.

17β-E2 resulted in values below the limit of quantification. These results suggest that there was little or no conversion of 17α-E2 to 17β-E2 in this study.

FURTHER CLINICAL DEVELOPMENT

Because mitochondrial function plays such a pivotal role in determining cell viability, mitoprotective strategies, as described here, are likely to provide therapeutic benefit in a host of chronic neurodegenerative diseases such as AD, PD, ALS, and FRDA, not to mention more acute cell death from stroke or myocardial infarction. However, any contention that mitoprotective therapeutics like 17α-E2 will yield clinically meaningful benefits remains to be tested in the clinic. The safety and PK studies described above are a reasonable start, but further clinical development of 17α-E2 would be facilitated by directly testing several inferences, including that it does not induce hormonal responses at the doses used and that it is indeed functioning as a lipophilic antioxidant *in vivo*.

The latter response has already been demonstrated for 17α-E2 in studies that monitored the lag-phase of plasma LDL oxidation,[84,89,90] which correspondingly serves to recommend this parameter as a convenient index of compound activity. Similarly, plasma measurements of prostaglandin F(2)–like compounds, such as F(2)-isoprostanes, are appropriate indices of activity

given that the likely mechanism of action for 17α-E2 is via moderation of lipid peroxidation. F(2)-isoprostanes are initially formed via nonenzymatic free radical–induced peroxidation of arachidonic acid and then released in free form, which are stable and present in detectable quantities.[91] As such, they are selective *in vivo* indicators of lipid peroxidation that not only increase dramatically in several animal models of oxidative injury but also are responsive to antioxidant treatments.[91]

Although plasma isoprostanes are informative indicators of oxidative stress, the evidence for them reflecting disease progression in neurodegenerative disorders is contradictory.[92–95] However, the next clinical development phase of 17α-E2 is not dependent on resolving whether plasma isoprostanes accurately reflect disease severity or progression but rather whether they are responsive to treatment with 17α-E2, as they are with vitamin C and E supplementation.[96] Thus, *ex vivo* LDL oxidation studies and plasma isoprostanes are viable indices of lipophilic antioxidant activity, if not yet of treatment efficacy in disease.

To evaluate whether 17α-E2 is hormonally active at pharmacologically relevant doses, hemostatic or inflammatory markers known to respond to 17β-E2 and HT could be monitored.[97,98] Plasminogen activator inhibitor type 1 (PAI-1) and fibrinogen are among the most responsive of the hepatic hemostatic parameters to oral 17β-estradiol treatments in healthy postmenopausal women, although the inflammatory marker C-reactive protein is also significantly responsive.[99–101] As such, any or all of these would be well-suited as indices of hormonal activity for 17α–E2.

In summary, data from an unusually broad range of cell and animal studies indicate that the cytoprotective effects of 17α-E2, and other estrogen analogues, can be separated from confounding, and potentially deleterious, hormonal activity associated with current oral HT regimes. However, the potential clinical utility of 17α-E2 would be advanced if several justified inferences could be examined, such as whether the compound is indeed nonhormonal at doses required to moderate lipid peroxidation *in vivo*. Once such issues are resolved, it will be possible to test whether therapeutic interventions designed to stabilize mitochondrial function and to correspondingly forestall cell death will yield tangible benefits to patients suffering from the extraordinarily wide range of degenerative diseases where mitochondrial failure contributes to etiology.

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