

Selective Estrogen Receptor Modulators (SERM) for the Brain: Recent Advances and Remaining Challenges for Developing a NeuroSERM™

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ABSTRACT Estrogen regulation of cognitive function and prevention of neurodegenerative disease has come to be of major scientific and clinical importance. While these functions rank paramount among concerns of women during menopause, the neoplastic risks associated with estrogen and hormone replacement therapy lead most women to elect against hormone intervention during menopause or to seek alternative estrogens in an attempt to ameliorate menopause-associated deficits and disease risks. Development of an effective selective estrogen receptor modulator (SERM) for use as an alternative to hormone replacement therapy must address the issues of efficacy in and availability to the brain. A brief review of estrogen effects on cognition, neuroprotective capability, and disease prevention is provided followed by an analysis of current knowledge regarding SERM efficacy in brain. Lastly, the challenges that remain for developing an effective NeuroSERM™ are considered. *Drug Dev. Res.* 56:380–392, 2002. © 2002 Wiley-Liss, Inc.

Key words: estrogen; SERM; nafoxidene; tamoxifen; phytoestrogen; neurodegeneration; Alzheimer's disease

THERAPEUTIC CHALLENGE

Of the age-associated maladies, neurodegenerative diseases are among the most devastating and costly because they typically have a prolonged gestational period until frank symptomology develops, followed by an equally slow rate of debilitation. Of these neurodegenerative diseases, Alzheimer's is perhaps the most devastating and costly, with victims living anywhere between 5–25 years following diagnosis. Alzheimer's disease (AD) is the most frequent cause of dementia and the leading cause of a loss in independent living and institutionalization [Birge, 1997; Whitehouse, 1997]. The care and treatment of persons afflicted with AD results in health care costs of about \$100 billion dollars per year in the United States alone [Birge, 1997; Whitehouse, 1997].

Age remains the greatest risk factor for developing AD [Whitehouse, 1997]. The increasing number of

persons vulnerable to developing AD is staggering when one considers that globally 800,000 people reach the age of 65 every month [Holden, 1996; Whitehouse, 1997]. At the turn of the new millennium, there were nearly 42 million women over the age of 50 in the US alone [North American Menopause Society, 2001]. Of these, more than 31 million women were over the age of 55 and by the year 2020 the number of US women over 55 is estimated to be close to 50 million. Currently,

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a woman's average life expectancy is estimated at 79.7 years. However, a woman who reaches the age of 54 can expect to live to the age of 84.3 years. Nearly two-thirds of the total US population will survive to age 85 or longer. Based on these data, women can anticipate spending one-third to one-half of their lifetime in the menopausal state. Worldwide, there are currently more than 470 million women age 50-plus and 30% of those are projected to live into their 80s [North American Menopause Society, 2001]. Because women have a longer life expectancy than men, it would be anticipated that the prevalence of women with AD would exceed that of men. A double danger, however, exists for women. The data indicate that at any given age women exhibit a higher incidence of AD than their age-matched male counterparts [Payami et al., 1996]. Based on current epidemiological data, of the 18 million American women in their 70–80s, 40–50% can be expected to manifest the histopathological changes of AD [Henderson, 2000].

ESTROGEN AND MEMORY FUNCTION

Changes in cognitive functioning have long been associated with menopause [Neurgaren and Kraines, 1965]. The relationship between memory function and estrogen was first noted clinically as women entering menopause frequently voiced complaints of memory and concentration difficulties [Sherwin, 1999]. The first basic science data to indicate a potential link between estrogen and memory function were the findings of Luine [1985], who found that 17 β -estradiol increased choline acetyltransferase activity which led to increased acetylcholine levels. This finding provided the impetus for studies of memory function in neurologically normal women and the use of estrogen replacement therapy in women with AD. In the mid-1980s, Sherwin [2000] and her group began a systematic analysis of the impact of estrogen loss and replacement on memory function. Results of these analyses demonstrated that verbal memory declined with the loss of estrogen but could be restored to premenopausal levels when estrogen replacement therapy was rapidly instituted following surgically induced menopause [Sherwin, 1988]. Recent studies by Resnick and colleagues [Maki et al. 2001] found that women receiving hormone replacement therapy perform better than those not receiving therapy on tests for verbal learning and memory, including encoding, consolidation, and retrieval. In an earlier cross-sectional human female study, Resnick et al. [1997] found that women who were current users of hormone replacement therapy performed significantly better on a visual memory test (which has been shown to predict the onset of AD) than women who had never received hormone

replacement therapy. As part of this study, a subset of the women who never used hormone replacement was studied longitudinally. At the initial memory test none of the women were using hormone replacement therapy. During the intervening 6 years between the first and second testing periods, a subset of these women began hormone replacement therapy independent of the study. At the follow-up 6-year analysis, the women who did not use hormone replacement therapy exhibited a significant decline in memory retention compared to their first test performance. In stark contrast, the women who independently began hormone replacement therapy during the intervening 6 years showed no decline in memory function relative to their first test performance 6 years earlier. While they were able to maintain memory function comparable to that exhibited at the first test (6 years prior) they did not achieve performance that matched the level of those women who had received hormone replacement therapy earlier in menopause. Considered together, these data indicate that estrogen replacement therapy can reverse memory deficits associated with menopause if begun soon after the initiation of menopause. Hormone replacement intervention initiated later in menopause is associated with a preservation of memory function but not a restoration to premenopausal levels.

ESTROGEN REPLACEMENT THERAPY AND RISK OF DEVELOPING AD

Multiple epidemiological studies indicate that estrogen/hormone replacement therapy can significantly reduce the risk of AD [Tang et al., 1996; Yaffe et al., 1998; Henderson, 2000]. These data, which were rapidly conveyed to the clinical and lay communities, stand as a unique and profoundly important series of observations. Estrogen replacement therapy was the first therapeutic intervention found to significantly reduce the risk of AD. Moreover, it remains the best characterized of several therapeutic interventions, such as nonsteroidal anti-inflammatory agents, proposed to reduce the risk of AD [Brinton and Yamazaki, 1998; Brinton, 1998].

If the epidemiological projections prove correct, the widespread use of estrogen replacement therapy during menopause could reduce the number of women in the US with AD by more than one million [Henderson, 2000]. Thus far, the human data are observational; however, several randomized, placebo-controlled, double-blind clinical trials are currently under way in the US and the UK. The best known of these is the Women's Health Initiative of the National Institutes of Health, which is a large-scale, long-term (follow-up for at least 6 years) study in which healthy women are randomized to receive conjugated equine estrogens (with or without a progestin) and monitored

for health endpoints (167,000 women age 50–79; 67,000 in clinical trials and 100,000 in observational studies) [NIH, 2002]. One arm of the Women's Health Initiative will evaluate the role of estrogen replacement therapy in 7,525 women on both symptom onset and progression of AD [Shumaker, 1996]. Results of this study will begin to emerge about the year 2006. A recent report from Brinton et al. [2000] has found that the form of estrogen replacement therapy, conjugated equine estrogens, used in the Women's Health Initiative is both neurotrophic and neuroprotective against toxic insults associated with AD in hippocampal, cortical, and basal forebrain neurons.

While the existing clinical data on estrogen replacement therapy and prevention of AD are very encouraging, the data on the treatment of women with existing disease are not. Because of the association of AD between loss of basal forebrain neurons that synthesize acetylcholine coupled with the findings of estrogen-induced increased in acetylcholine synthesis, Fillit et al. [1986] investigated the impact of estrogen replacement therapy on cognitive function in women with AD. Results of this small clinical trial proved to be pivotal and led to multiple international clinical and epidemiological studies which in turn led to intense study of the impact of estrogen on mechanisms of memory both in animals and humans [Brinton, 2001]. Despite the positive benefits reported in earlier small, open-label clinical trials [Birge, 1997], two randomized, double-blind, placebo-controlled, parallel-group trials found that both short-term [Henderson et al., 2000] and long-term (1 year) estrogen therapy [Mulnard et al., 2000] did not improve symptoms of most women with AD. Thus, it would appear that estrogen maintains and sustains neuronal viability to prevent degenerative disease, whereas it appears to be ineffective in reversing degenerative disease process.

WHY THE NEED FOR A NEUROSERM™?

Despite the encouraging epidemiological data indicating a reduced risk of developing AD in women who have ever received estrogen replacement therapy [Yaffe et al., 1998], only 25% of eligible postmenopausal women elect to receive prescribed estrogen replacement therapy and of that number ~50% discontinue use within the first year of therapy and greater than 70% of those for whom it has been prescribed are not compliant [Hammond, 1994]. Moreover, only 20% of women prescribed estrogen are still compliant 3 years later [Medicine, 2002]. The principle reason women forego estrogen replacement therapy is the fear of developing breast cancer [Hammond, 1994]. And therein lies the challenge.

One strategy to address this challenge is to develop estrogen alternatives that exert estrogen agonist properties in brain and bone while exerting estrogen antagonist action in the breast and uterus. Several such compounds that exert a mixed estrogen receptor agonist/antagonist profile have been developed, such as tamoxifen and raloxifene [Grese et al., 1998; Gustafsson, 1998]. Many more are in the pipeline. SERMs and estrogen agonist molecules that are currently available and currently in development are shown in Table 1.

The question of whether estrogen alternatives such as phytoestrogens and selective estrogen receptor modulators (SERMs) are effective estrogens for the promotion of memory function and the prevention of degenerative disease in postmenopausal women remains unanswered and will remain unanswered for decades due to the long periods of observation necessary to determine a reduced risk of developing AD. However, a body of data is emerging that has the potential to predict therapeutic efficacy of SERMs in

TABLE 1. Estrogens and SERMs of Historical and Current Interest

| Estrogen-related Steroids | Triphenylethylene Derivatives | Benzothiophene Derivatives | Dihydronaphthalene Derivatives | Tetrahydro-naphthalene Derivatives | Benzopyran Derivatives | Pure Steroidal Antiestrogens |
|---------------------------|--------------------------------|----------------------------|--------------------------------|------------------------------------|------------------------------------|------------------------------|
| 17 beta-Estradiol | Tamoxifen (Nolvadex) | Raloxifene (Evista) | Trioxifene | Lasofloxifene (CP336156) | Levomeloxifene (Levomeloxifene) | Fulvestrant (ICI 182,780) |
| 17 alpha-Ethinylestradiol | Toremifene (Fareston) | Arzoxifene (LY353381-HCl) | Nafoxidene | | Centchroman (Ormeloxifene) | ICI 164,384 |
| 17 alpha-Dihydroequilenin | Droloxifene | | | | EM800 (SCH57050) | |
| | Clomifene (Clomid; Seraphene) | | | | EM652 (active metabolite of EM800) | |
| | Miproxifene phosphate (TAT-59) | | | | | |
| | Idoxifene | | | | | |
| | GW 5638 | | | | | |

brain. An effective SERM for the brain, a NeuroSERM™, would exert estrogen agonist action in brain and bone, and estrogen antagonist action in breast and uterus. The ideal NeuroSERM™ would also exert estrogen agonist action in the vagina and a null ligand effect on factors regulating coagulation (Fig. 1).

In discussing estrogenic effects in brain, most attention is focused on its impact in the female brain. However, estrogen effects in the male brain are of similar importance, as estrogen is equally effective in activating mechanisms of memory and neuroprotection in neurons from the male brain as they are in the female brain [Foy et al., 1999; Bi et al., 2000, 2001]. Thus, an effective NeuroSERM™ that lacks feminizing effects should be efficacious in promoting the benefits of estrogen in brain for both women and men.

PREDICTIVE CAPABILITY OF BASIC SCIENCE ANALYSES OF ESTROGEN ACTIVATION OF MEMORY AND NEUROPROTECTIVE MECHANISMS AS PREDICTORS OF THE THERAPEUTIC EFFICACY OF SERMS IN BRAIN.

While the full spectrum of mechanisms that underlie memory function remain to be elucidated, a number of cellular responses have been well char-

acterized as essential components of long-term memory function [Brinton, 2001]. Two features are particularly relevant for NeuroSERM™ efficacy in brain. The first requirement is the promotion of neuronal process outgrowth from brain regions involved in memory function and the generation of new synapses in brain [Greenough and Bailey, 1988] via potentiation of the glutamate NMDA receptor, which is well-documented to be one biochemical strategy to activate memory formation [Brinton, 2001]. Thus far, select estrogens, 17 β -estradiol, equilin, and the complex formulation of conjugated equine estrogens have been shown to promote neuronal process outgrowth [Brinton, 1993; Brinton et al., 1997a,b, 2000] and potentiate glutamate NMDA receptor function [Foy et al., 1999; Nilsen et al., 2002]. The in vitro neurotrophic effects of select estrogens in cultured neurons have been paralleled by in vivo analyses which documented 17 β -estradiol induction of an increase in both dendritic spines and synapses [Woolley, 1999]. Moreover, the estrogen-induced increases in neuronal morphology were dependent on the NMDA receptor [Brinton, 2001]. 17 β -estradiol significantly potentiated glutamate NMDA receptor-mediated excitatory postsynaptic potentials [Foy et al., 1999] and significantly

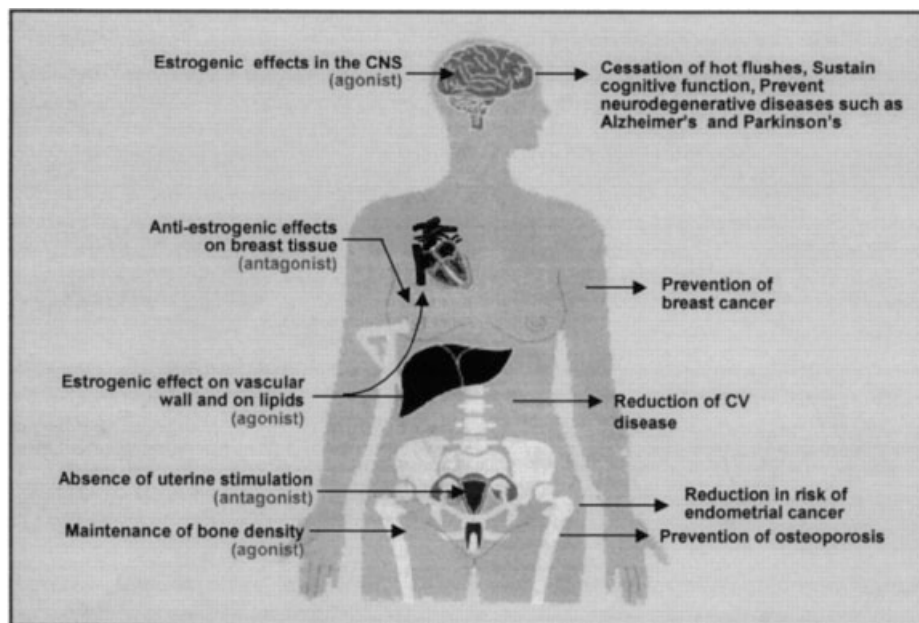


Fig. 1. Goals of a NeuroSRM™. An effective NeuroSERM™ must induce a cessation in the hot flashes associated with estrogen deficiency. In addition, NeuroSERM™ must promote memory function and prevent neurodegenerative diseases such as Alzheimer's disease. An effective estrogenic molecule for the brain must also consider other estrogen-responsive organs such as bone and the cardiovascular system. Thus far, most SERMs are developed as estrogen agonists in bone for prevention of osteoporosis. Results of

basic science analyses and human trials have indicated that an estrogen agonist effect of a SERM in bone does not predict its efficacy in brain. In fact, tamoxifen and raloxifene, both partial estrogen agonists in bone, are well documented to exert an adverse CNS effect in that they increase the frequency of hot flashes (Anthony et al., 2001). These data would indicate that brain sites of estrogen action respond preferentially to the antagonist effects of molecules with partial agonist/partial antagonist properties.

potentiated the level of intracellular calcium following activation of the NMDA receptor [Nilsen et al., 2002]. Thus far, a one-to-one correlation exists between the ability to potentiate neuronal process outgrowth, NMDA receptor function, and induction of memory function. Based on these cellular markers of estrogen action, it is possible to predict the efficacy of a molecule to act as a full estrogen agonist to induce neuronal mechanisms of memory function.

The second requirement for an effective NeuroSERM™ is the ability to promote neuronal defense mechanisms that protect against degenerative insults (Fig. 2). Several biochemical mechanisms have been proposed to underlie estrogen-inducible neuroprotection against toxic insults, such as beta amyloid and glutamate excitotoxicity. The best-known of these is estrogen activation of the MAP kinase-signaling pathway leading to an increase in phosphorylated extracellular-signal regulated kinase 1 (ERK1), a component of the mitogen-activated protein kinase (MAPK) pathway. In brain, estrogen activation of the MAP kinase signaling pathway is an obligatory component for estrogen-induced neuroprotection against a number of neurodegenerative insults [Singer et al., 1999; Singh et al., 1999; Wise et al., 2001; Nilsen and Brinton, 2002a,b].

The downstream mechanisms by which MAP kinase activation leads to neuroprotection rely on activation of the transcription factor CREB, increase expression of the antiapoptotic protein Bcl-2, and regulation of calcium sequestration by mitochondria [Nilsen and Brinton, 2001; Nilsen and Brinton, 2002a]. Another estrogen-activated pathway that potentially interacts with the estrogen-inducible MAP kinase signaling and confers neuroprotection is the Akt/protein kinase B pathway. Recently, Singh [2001] reported estradiol activation of the Akt/Protein kinase B kinase, which can mediate antiapoptotic signaling through increased expression of the antiapoptotic protein Bcl-2. Activation of these signaling cascades provide a unifying neuroprotective pathway that could lead to a reduced risk of AD.

IMPACT OF THE SERM TAMOXIFEN AND METABOLITE 4-HYDROXY-TAMOXIFEN ON NEURONAL SURVIVAL AND CORRELATES OF MEMORY

Based on the estrogen-induction of predictive neuronal markers of memory and neuroprotection, we pursued whether the SERM tamoxifen or its active metabolite, 4-hydroxy-tamoxifene, exerted estrogenic

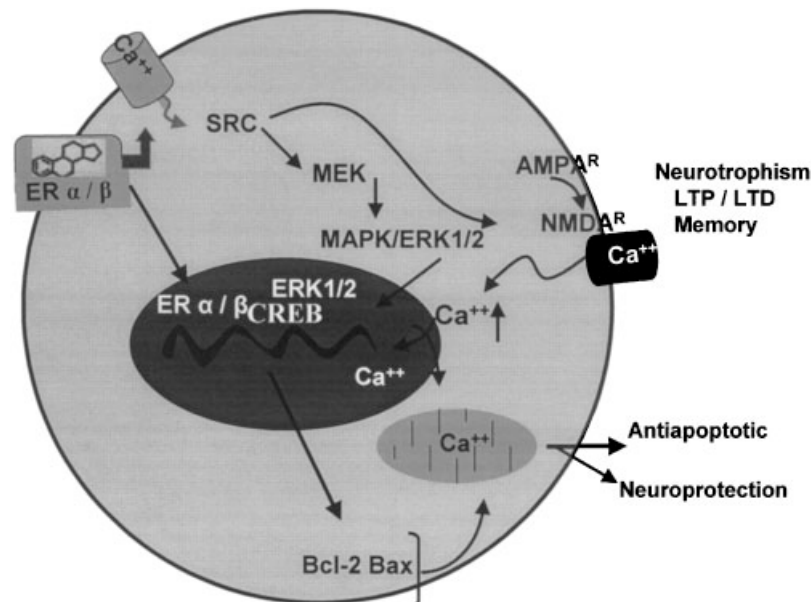


Fig. 2. Estrogen-induced signaling in neurons. An effective NeuroSERM™ must activate mechanisms of memory and neuroprotection. The data thus far indicate that the signaling cascade leading to estrogen promotion of memory mechanisms and neuroprotection is mediated by a plasma membrane site of action that leads to activation of the Src/MAP kinase signaling cascade. The proposed model of estrogen action is the transient activation of a calcium channel that increases intracellular calcium to activate the tyrosine kinase Src. Src then activates the MAP kinase signaling cascade that leads to phosphorylation of the glutamate NMDA receptor, necessary for

enhancement of intracellular calcium levels and subsequent activation of long-term memory mechanisms. This same signaling cascade leads to activation of neuroprotective mechanisms that include translocation of the MAP kinase ERK1/ERK2 to the nucleus and subsequent activation of the transcription factor CREB to increase expression of the antiapoptotic gene *bcl-2*. The Bcl-2 protein will prevent mitochondrial dysfunction associated with neurotoxic insults linked to neurodegenerative diseases such as Alzheimer's. Activation of this signaling cascade is predicted to be a necessary requirement for an effective NeuroSERM™.

agonist action in neurons derived from brain regions required for memory function and vulnerable to AD. Tamoxifen is a triphenylethylene, nonsteroidal mixed antagonist/agonist SERM that is a competitive antagonist at the estrogen receptor (ER) [Willson et al., 1994; Jordan and Koerner, 1975; Coezy et al., 1982]. The SERM tamoxifen and its active metabolite, 4-hydroxy-tamoxifen, are examples of substituted triphenylethylene (anti)estrogens that were developed by successive chemical modifications of a triphenylethylene core, formed by the addition of an extra phenyl ring to a stilbene nucleus [Kuiper et al., 1997]. Major circulating metabolites of tamoxifen include N-desmethyl-tamoxifen and 4-hydroxy-tamoxifen, both of which have antiestrogenic activity [Jordan, 1983]. N-desmethyl-tamoxifen is the principal serum metabolite and has a half-life twice that of tamoxifen, but is slightly less potent [Catherino and Jordan, 1993]. 4-Hydroxy-tamoxifen is a minor metabolite with a much shorter half-life, but has a binding affinity to estrogen receptor α (ER α) 20–30 times greater than that of tamoxifen and equivalent to the affinity of estradiol [Jordan et al., 1977; Eppenberger et al., 1991; Grill and Pollow, 1991; Kawamura et al., 1991; Bruning, 1992].

Tamoxifen was first developed in 1962 as a birth control pill [Harper and Walpole, 1967] and since 1971 has been used to treat breast cancer in postmenopausal women [Cole et al., 1971]. Results from an NIH study “Breast Cancer Prevention Trial,” concluded in 1998, found that Nolvadex (trade name for tamoxifen) reduced breast cancer by up to 45% in women at high risk for the disease but also showed an increase in endometrial cancer [Fisher et al., 1998]. In 1999, the American Society for Clinical Oncology recommended prescribing tamoxifen for the prevention of breast cancer [Gerber and Krause, 1999].

Tamoxifen competes with estrogen for binding to the estrogen receptor and a number of the biological effects of tamoxifen can be reversed by estrogen [Jordan and Koerner, 1975; Lippman and Bolan, 1975]. However, not all biological effects of tamoxifen can be reversed by estrogen, which suggests that a portion of tamoxifen effects are not mediated through the estrogen receptor [Jordan and Koerner, 1975; Lippman and Bolan, 1975; Reddel et al., 1983; Sudo et al., 1983; Sutherland et al., 1983]. Also, tamoxifen can bind to a high-affinity site, which is distinct from the estrogen-binding site of the estrogen receptor, but it is not known whether this site mediates any biological effects of tamoxifen [Das et al., 1993]. Specifically, the biology of tamoxifen has indicated that it functions as an antagonist in most tissue environments but displays agonist activity in bone, liver, and uterus [Mitlak and Cohen, 1997; Shang and Brown, 2002].

We sought to determine whether tamoxifen and 4-hydroxy-tamoxifen would function as an estrogen agonist in neurons to promote neuronal survival and memory mechanisms [O'Neill and Brinton, 2001, 2002]. Biochemical analysis and direct measurement of cell viability revealed that both tamoxifen and 4-hydroxy-tamoxifen were modestly neuroprotective over a range of therapeutically relevant concentrations (5–1,000 ng/ml). Analysis of hippocampal neuron viability and survival indicated that both tamoxifen and 4-hydroxy-tamoxifen were modestly neuroprotective against beta amyloid and glutamate-induced toxicity [O'Neill and Brinton, 2001, 2002]. To determine whether tamoxifen and 4-hydroxy-tamoxifen promoted neuronal process outgrowth, a morphological correlate of memory, neurons from three brain regions involved in memory function and affected in AD—cortex, hippocampus, and basal forebrain—were assessed. Results of these analyses indicated that neither tamoxifen nor 4-hydroxy-tamoxifen promoted neuronal process outgrowth, as determined by six morphological parameters [O'Neill and Brinton, 2001, 2002]. Because neurotrophic estrogens potentiate the NMDA receptor glutamate-mediated rise in intracellular calcium, ratio-metric fluorescent calcium-imaging analyses using the calcium-sensitive dye Fura-2 were conducted. Results of these analyses confirmed that neither tamoxifen nor 4-hydroxy-tamoxifen potentiated the intracellular calcium response to glutamate, as has been demonstrated for neurotrophic estrogens. Moreover, 4-hydroxy-tamoxifen exerted antagonist actions to block estradiol potentiation of the glutamate-induced rise in intracellular calcium [O'Neill and Brinton, 2001, 2002]. Results of these analyses indicate that tamoxifen and its active metabolite 4-hydroxy-tamoxifen exert modest neuroprotective effects but do not promote cellular features and mechanisms of memory formation. These data support a partial and modest estrogen agonist property of these SERMs but indicate that in the presence of the full agonist tamoxifen and its active metabolite 4-hydroxy-tamoxifen act as full estrogen receptor antagonists (see Table 2 for summary).

A recent clinical report by Paganini-Hill and Clark [2001] support the predictions of our basic science data. Women who used tamoxifen for 5 years or more were found to perform more poorly on a series of cognitive tests than women who had used the drug for shorter periods.

IMPACT OF THE SERM RALOXIFENE ON NEURONAL SURVIVAL AND CORRELATES OF MEMORY

The most recently FDA-approved molecule for the treatment of osteoporosis is raloxifene, a nonsteroidal benzothiophen derivative that binds

TABLE 2. Impact of SERMs on Neuronal Markers of Memory and Neuroprotection

| SERM | Memory correlate | Plasma membrane integrity | Marker of neuronal viability |
|---------------------|------------------|---------------------------|------------------------------|
| Tamoxifen | Ineffective | Modest protection | Ineffective |
| 4-Hydroxy-tamoxifen | Ineffective | Modest protection | Ineffective |
| Raloxifene | Modest efficacy* | Modest protection | Ineffective |
| Phytoestrogens | Ineffective | Modest protection | Ineffective |

*Raloxifene induced neuronal markers of memory at a single concentration in a single brain region involved in memory function. However, the concentration required to induce the effect is unlikely to be achieved in brain. Details are contained within sections devoted to each SERM.

with high affinity to the nuclear estrogen receptor [Yang et al., 1996; Brzozowski et al., 1997; Delmas et al., 1997; Baker et al., 1998]. Raloxifene has a mixed pharmacological profile, acting as both an estrogen receptor antagonist and agonist, depending on the tissue [Brzozowski et al., 1997; Wijayarathne et al., 1999]. In the breast and uterus, raloxifene acts as a classical antiestrogen to inhibit the growth of mammary or endometrial carcinoma [Purdie and Beardsworth, 1999]. In nonreproductive tissues, raloxifene acts as a partial estrogen agonist to prevent bone loss and lower serum cholesterol, with a pharmacological profile similar to that of 17 β -estradiol in both ovariectomized rats and postmenopausal women [Sato et al., 1996; Delmas et al., 1997; Purdie and Beardsworth, 1999].

Studies conducted in neural preparations show that raloxifene exerted mixed agonist/antagonist effects. In the rat hypothalamus, raloxifene was found to increase dopamine content two-fold, acting as a partial agonist, but was able to block the five-fold induction of dopamine levels by estradiol, acting as a full antagonist [Grandbois et al., 2000]. In human females, raloxifene significantly increased hot flashes, suggesting that raloxifene acts as an estrogen receptor antagonist on sites regulating vasomotor function [Cohen and Lu, 2000]. In studies using a rat pheochromocytoma cell line (PC12), raloxifene showed estrogen agonist properties to increase process outgrowth when the PC12 cells were pretreated with NGF [Nilsen et al., 1998]. Genazzani et al. [1999] found that raloxifene exerted estrogen-like action on neuroendocrine opiate-ergic pathways when administered alone in ovariectomized rats, which was reversed in fertile or in ovariectomized rats treated with 17 β -estradiol, where it exerted an antiestrogen effect. Wu et al. [1999], investigating the impact of raloxifene alone or in combination with estradiol on choline acetyltransferase activity, found that ovariectomized female rats treated with either estradiol benzoate or raloxifene showed an increase in choline acetyltransferase activity.

We sought to determine whether the SERM raloxifene exerted estrogen agonist or antagonist effects in neurons involved in memory function and adversely

affected in AD. Low concentrations of raloxifene significantly reduced a marker of basal membrane damage and had no deleterious on neuronal survival. High concentrations of raloxifene (1,000–5,000 ng/ml) induced a significant increase in plasma membrane damage and a significant decrease in neuronal survival. At subtoxic concentrations, raloxifene induced significant neuroprotection against β -amyloid_{25–35}, hydrogen peroxide, and glutamate-induced toxicity. Results of analyses to determine whether raloxifene acted competitively or synergistically with 17 β -estradiol revealed that a postmenopausal level of 17 β -estradiol exerted a significantly greater increase in neuronal survival against β -amyloid and glutamate-induced toxicity compared to 50 ng/ml raloxifene. The combined presence of raloxifene and 17 β -estradiol was significantly neuroprotective against β -amyloid_{25–35} and glutamate-induced excitotoxicity, but was significantly lower than 17 β -estradiol alone, while not significantly different than raloxifene alone. Results of this study indicate that raloxifene exerted partial to full estrogen agonist action in the absence of 17 β -estradiol, whereas in the presence of 17 β -estradiol raloxifene exerted a mixed estrogen agonist/antagonist effect. Morphologic analyses demonstrated that raloxifene significantly increased neuronal outgrowth of hippocampal neurons within a narrow dose range, which was blocked by a glutamate NMDA receptor antagonist. Raloxifene did not promote process outgrowth of basal forebrain or cortical neurons (Table 2). Together, these data indicate that raloxifene can act as a partial estrogen agonist in the absence of 17 β -estradiol and acts as a partial antagonist in the presence of 17 β -estradiol.

What is the predictive significance of these findings for memory function and prevention of neurodegenerative disease? The ability of raloxifene to promote neuronal mechanisms of memory function in the hippocampus suggests that raloxifene could act as an estrogen agonist to promote short-term memory function within the hippocampus. However, the dose-response curve for raloxifene-induced hippocampal neuron outgrowth would predict that any estrogenic effect would be highly dose-dependent and that

the range of efficacious concentrations would be very narrow. The lack of an effect in cortical neurons suggests that the select functions subserved by estrogen-induced neuronal outgrowth would not be activated by raloxifene. The other major problem is availability of raloxifene to the brain.

The neuroprotective effects of raloxifene would predict that some beneficial effect would be achieved from low doses of raloxifene, but not from higher doses. The magnitude of the neuroprotection varied across the neuronal populations investigated and ranged from a 10–50% reduction in markers of neuronal damage, depending on the neuronal population. It remains to be determined whether this magnitude of neuroprotection could lead to a decreased risk of developing AD, as has been found with estrogen replacement therapy. The data would further predict that raloxifene could act as a partial estrogen agonist in hippocampus to promote memory function but that the concentration required to promote that effect very likely exceeds that which can cross the blood–brain barrier and that the range of effective concentrations would be very narrow. These predictions were borne out in a large, randomized clinical trial of raloxifene in women with osteoporosis who also underwent cognitive testing. After 3 years there were no differences between the raloxifene and placebo groups in the frequency of cognitive decline or in the occurrence of dementia [Yaffe et al., 2001]. Both the raloxifene-treated group and the placebo group showed cognitive decline over the time frame of the study. Results of these studies indicated that raloxifene can act as a partial estrogen agonist in the absence of 17 β -estradiol while exerting a partial antagonism in the presence of 17 β -estradiol. While the concentration of raloxifene in brain achieved with the 60-mg therapeutic dose remains to be determined, it is evident that sufficient quantities of raloxifene can cross the blood–brain barrier to act as an estrogen receptor antagonist, leading to an increase in the frequency of hot flashes, a hypothalamic effect of estrogen receptor antagonists [Cohen and Lu, 2000].

IMPACT OF PHYTOESTROGENIC SERMS ON NEURONAL SURVIVAL AND CORRELATES OF MEMORY

Phytoestrogens are plant-derived molecules that structurally resemble endogenous estrogens containing a diphenolic chemical structure that can directly bind to estrogen receptors (ER) to regulate gene expression mediated by estrogen response element [Kurzer and Xu, 1997]. Select phytoestrogens have been found to exhibit some estrogen agonist-like properties [Makela et al., 1995; Stahl et al., 1998]. However, phytoestro-

gens also act as partial estrogen receptor antagonists [Bowers et al., 2000]. Cellular mechanisms activated by phytoestrogens are diverse and appear to be concentration-dependent [Wang and Kurzer, 1997]. For example, at low concentrations phytoestrogens can induce proliferation of ER-positive MCF-7 cells but not of ER-negative MDA-MB-231 cells [Wang and Kurzer, 1997]. Conversely, several studies have shown antiproliferative effects of phytoestrogens on human breast cancer cell lines and in animal experiments. High concentrations of phytoestrogens (comparable to those achieved in plasma following consistent soy consumption) can significantly inhibit proliferation of human breast carcinoma cell growth [Wang and Kurzer, 1997; Zava and Duwe, 1997; Dixon-Shanies and Shaikh, 1999; Balabhadrapathruni et al., 2000].

One mechanism proposed to account for the inhibitory action of phytoestrogens is competitive inhibition with endogenous estrogens through binding to the ERs. Indeed, some phytoestrogens competitively suppress the binding of 17 β -estradiol to ERs in human mammary tumor tissue [Kuiper et al., 1998; Nikov et al., 2000]. Coumestrol has been demonstrated to antagonize the neuroendocrine actions of estrogen in the brain and pituitary via ER α [Jacob et al., 2001]. Recent data indicate that phytoestrogens exhibit a greater affinity for ER β relative to ER α [Kuiper et al., 1998]. This finding is of interest since ER β has a higher level of expression than that of ER α in brain regions critical to memory function and which are vulnerable to AD, such as the basal forebrain, hippocampus, and cerebral cortex. Other potential mechanisms of phytoestrogen action is through tyrosine kinase inhibition, DNA topoisomerase inhibition, inhibition of aromatase function, stimulation of sex-hormone-binding globulin in the liver thereby reducing free, biologically active estradiol in the plasma and by inducing antioxidant properties [Adlercreutz et al., 1993; Monti and Sinha, 1994; Kulling and Metzler, 1997; Pino et al., 2000]. Because of their mixed agonist/antagonist estrogen receptor profile, phytoestrogens have received considerable attention as potential alternatives to estrogen.

To determine whether phytoestrogens exert estrogen agonist effect in neural tissue, we investigated the neuroprotective and neurotrophic efficacy of six phytoestrogens: genistein, genistin, daidzein, daidzin, formononetin, and equol. Results of these studies demonstrated that all phytoestrogens induced a modest but significant reduction in plasma membrane damage following exposure to glutamate and β -amyloid_{25–35}. However, none of the phytoestrogens induced a significant increase in a marker of neuronal viability, whereas the full estrogen agonist 17 β -estradiol both decreased membrane damage and increased markers

of neuronal viability. Analysis of the neurotrophic potential of genistein and daidzein, two phytoestrogens that exerted a significant reduction in plasma membrane damage, demonstrated that neither of these molecules promoted hippocampal neuron process outgrowth. Results of these analyses indicate that, although phytoestrogens exert a neuroprotective effect at the plasma membrane, they do not sustain neuronal viability nor do they induce cellular correlates of memory (Table 2). Data derived from these investigations would predict that phytoestrogens could exert modest neuroprotection analogous to that of antioxidants but that these molecules are not functional equivalents to endogenous 17 β -estradiol or to estrogen replacement formulations and therefore would be unlikely to reduce the risk of AD or to sustain memory function in postmenopausal women.

The behavioral data on phytoestrogens and cognition are variable. In animal studies, Clarkson and co-workers [Pan et al., 2000] found that a soy diet improved working memory in the ovariectomized retired breeder female rats. Furthermore, their results indicated that a soy diet did not antagonize the beneficial effects of estradiol on the working memory of these rats [Pan et al., 2000]. However, these studies did not control for the impact of the nonphytoestrogen component of the diet. Although their findings are encouraging, studies in humans are not consistent with the animal data. Rice et al. [1995] studied Japanese-American women 65 years or older living in King County, Washington, to determine the impact of soy consumption and cognitive function. Women using estrogen replacement therapy but who consumed tofu more than three times per week were not protected against cognitive impairment. On the other hand, women using estrogen replacement therapy who consumed tofu less than three times per week were less likely to be cognitively impaired than were nonestrogen users. These data suggest that in women already using ERT consumption of high amounts of tofu can block the beneficial effects of ERT, thereby acting as estrogen antagonists. Furthermore, a recent Honolulu-Asia Aging study by White et al. [2000] showed a correlation between mid-life consumption of tofu with late-life cognitive impairment and dementia. Thus, it would appear that effects of phytoestrogens on cognition are multifaceted, depending on the regime and dose of phytoestrogen consumption.

To date, there is no evidence that a soy-based diet protects against AD, as the prevalence of AD in Asian countries, such as China and Japan, have rates of AD similar to rates cited for the US [Ogura et al., 1995]. Suh and Shah [2001] found that overall

prevalence rates for AD and vascular dementia were equal in men, but AD was predominant in women. In fact, AD has become nearly twice as prevalent as vascular dementia in Japan, China, and Korea [Suh and Shah, 2001].

REMAINING CHALLENGES FOR DEVELOPING A NEUROSERM™

Based on the results from predictive basic science model systems and behavioral trials in both animals and humans, the preponderance of data indicate that neither tamoxifen, raloxifene, nor phytoestrogens fulfill all the criteria for full estrogen agonists in brain [Brinton et al., 1998; O'Neill and Brinton, 2001; Zhao, 2002]. While great strides have been made in developing effective SERMs for bone, the challenge of developing an effective estrogen alternative for the brain remains. Successful development of a NeuroSERM™ is predicated in part upon fully understanding the sites and mechanisms of estrogen action in brain that lead to maintenance of memory function and prevention of neurodegenerative disease.

The complexity of the mechanisms required for estrogen-induced neurotrophism and neuroprotection are exemplified in the signaling cascade induced by neuroprotective estrogens (see Fig. 2 for schematic). First, the existing data indicate that both the neurotrophic and neuroprotective effects of full estrogen agonists are dependent on activation of the Src/MAP kinase signaling cascade [Nilsen et al., 2002]. Estrogen activation of the Src/MAP kinase signaling cascade can lead to regulation of gene expression, as is the case for the antiapoptotic gene *bcl-2*, very likely through MAP kinase activation of the transcription factor CREB [Nilsen and Brinton, 2002]. Surprisingly, activation of this signaling cascade is mediated by estrogen receptors which antigenically resemble the nuclear estrogen receptor proteins estrogen receptor alpha ($ER\alpha$) and beta ($ER\beta$), but are located at the plasma membrane. While there is evidence that both $ER\alpha$ or $ER\beta$ can activate MAP kinase [Wade et al., 2000], it remains to be determined whether $ER\alpha$ or $ER\beta$ or both are required for activation of the Src/MAP kinase signaling cascade in neurons. Thus far, the preponderance of data point to estrogen-inducible signaling cascades initiated at the plasma membrane as those crucial for estrogen-associated memory function and neuroprotection against neurodegenerative disease.

While the essential mechanisms of estrogen regulation of cognition and neuronal defense are initiated at a membrane receptor for estrogen, we can anticipate that nuclear sites of estrogen action will

impact neuronal and glial function. We know little regarding the expression of estrogen receptor coregulator proteins in neurons or glia. Of the estrogen-inducible genes beyond those of the reproductive hypothalamic/pituitary axis, the best-characterized is glial fibrillary acidic protein in astrocytes which contains an estrogen response element [Stone et al., 1998]. Current advances in the molecular characterization of nuclear estrogen receptors and the coregulatory proteins that regulate estrogen receptor function in the nucleus has provided a firm foundation upon which to explore these complexities in neural tissue and to determine the extent to which these interactions in the nucleus affect estrogen effects on cognition and protection against neurodegenerative disease [Katzellenbogen et al., 2000; Kraichely et al., 2000]. Moreover, emerging proteomic strategies will prove pivotal in determining both mechanisms and downstream consequences of estrogen action in brain. Armed with current investigative technologies and a broader understanding of the multifaceted nature of estrogen action in brain, we are poised to design molecules that are accessible to brain and specifically target estrogen-associated mechanisms at the plasma membrane crucial for sustaining cognitive health and function in both women and men.

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