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Targeted Brain Delivery of 17ß-Estradiol Via Nasally Administered Water Soluble Prodrugs

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ABSTRACT The utility of the nasal route for the systemic delivery of 17ß-estradiol was studied using watersoluble prodrugs of 17ß-estradiol. This delivery method was examined to determine if it will result in preferential delivery to the brain. Several alkyl prodrugs of 17ß-estradiol were prepared and their physicochemical properties were determined. In vitro hydrolysis rate constants in buffer, rat plasma, and rat brain homogenate were determined by high-performance liquid chromatography. In vivo nasal experiments were carried out on rats. Levels of 17ß-estradiol in plasma and cerebral spinal fluid (CSF) were determined with radioimunoassay using a gamma counter. The study revealed that the aqueous solubilities of the prodrugs were several orders of magnitude greater than 17ß-estradiol with relatively fast in vitro conversion in rat plasma. Absorption was fast following nasal delivery of the prodrugs with high bioavailability. CSF 17ß-estradiol concentration was higher following nasal delivery of the prodrugs compared to an equivalent intravenous dose. It was determined that water-soluble prodrugs of 17ß-estradiol can be administered nasally. These prodrugs are capable of producing high levels of estradiol in the CSF and as a result may have a significant value in the treatment of Alzheimer's disease.

Key Words: Nasal delivery, CNS delivery, 17ß-estradiol; Alzheimer's disease, Prodrugs.

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INTRODUCTION

Estradiol, the primary estrogenic hormone produced by the human ovary, has been used in substitution therapy in menopausal women or in cases of steroidogenesis failure. It has also been used as a contraceptive and as a hypocholesteremic drug. Recently it has been found that estrogen intake may delay the onset and decrease the risk of Alzheimer's disease [1]. Estrogen has been shown to be important for the differentiation of certain nuclei of the brain [2] and recent evidence suggests that estrogens may be important for normal brain function throughout life [3].

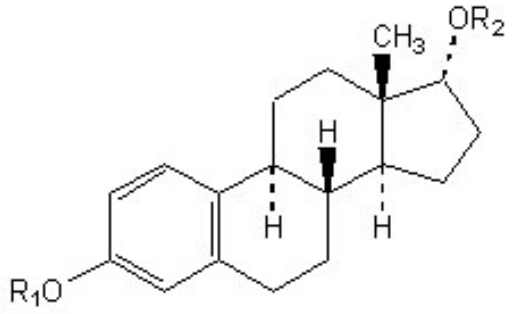
Although the oral route is the most convenient method of administration, estradiol is inactivated by first pass metabolism in the gastrointestinal tract and liver. It has been shown that more than 95% of an oral dose of 17ß-estradiol is converted to metabolites before it reaches the blood [4]. For this reason, oral estradiol has not been used extensively. Attempts to improve the oral bioavailability of estradiol by using prodrugs have not been successful [5].

Transdermal administration of estradiol was found to be superior to the oral route and transdermal dosage forms are currently on the market. However, these are expensive and frequently cause skin irritation.

Nasal administration of estradiol in rats resulted in plasma levels of the drug similar to those following intravenous administration [6]. And, it has been established that the rat is an excellent animal model to study nasal absorption of drugs. For most nonpeptide drugs, the results obtained in the rat can

accurately predict the absorption profiles of drugs in humans [7]. In addition, some drugs when administered nasally to the rat resulted in CSF and olfactory bulb levels considerably higher than those following intravenous administration [7-11]. The passage of drugs from the nasal cavity to the CSF via the olfactory epithelium has been known for some time; the detailed mechanism of such a transfer has been discussed in a recent publication in some detail. [12]. The above observations would suggest that the nasal route could offer an attractive method for administration of estradiol.

Unfortunately, estradiol is not very water soluble, which makes the nasal administration of an effective dose (ie, 0.1 mg in a volume of 0.1 mL) impractical. On the other hand, watersoluble prodrugs that hydrolyze to estradiol in biological fluids might overcome this problem. Accordingly, it was hypothesized in this study that the nasal administration of watersoluble prodrugs of estradiol (Figure 1) should offer an attractive method for the delivery of estradiol into the systemic circulation and the brain for the prevention of Alzheimer's disease (AD).



3-DMABE$_2$ HCl: R_1=CO-$(CH_2)_3$-N-$(CH_3)_2$ HCl
R_2= H
3-DEAPE$_2$ HCl: R_1 =CO- $(CH_2)_2$-N-$(C_2H_5)_2$ HCl
R_2 =H
3-TMABE$_2$ iodide: R_1=CO- $(CH_2)_3$-N^+- $(CH_3)_3$ iodide
R_2 =H
17-DMABE$_2$ HCl: R_1 =H
R_2 =CO-$(CH_2)_3$- N-$(CH_3)_2$ HCl

Figure 1 - Chemical structure of 17β-estradiol and its ester prodrugs.

MATERIALS AND METHODS

Materials

17ß-estradiol and iodomethane were purchased from Sigma Chemical Company (St Louis, MO). 4-(Dimethylamino) butyric acid hydrochloride, 3-(dimethyleamino) propionic acid hydrochloride, oxalylchloride, sodium chloride, triethylamine, sodium acetate, and thionyl chloride were purchased from the Aldrich Chemical Company (Milwaukee, WI). All other chemicals and solvents were of high purity and were used as received from Fisher Scientific Company (Pittsburgh, PA).

Synthesis of 17ß-Estradiol Esters

4-(Dimethylamine) butyric acid hydrochloride (2.0 g, 0.012 mol) or 3-(dimethylamine) propionic acid hydrochloride (2.2 g, 0.012 mol) was used as a starting material. The amino acid was refluxed gently with oxalyl chloride (1.6 mL, 0.018 mol) for a short period of time until a clear yellow solution was formed. The solution mixture was then flushed very gently with a stream of nitrogen to remove excess oxalyl chloride leaving a solid behind (the acid chloride).

The phenolic esters, 3-N, N-dimethylamino butyl ester hydrochloride (3-DMABE$_2$HCl), 3-N, N-diethylamino propionyl ester hydrochloride (3-DEAPE$_2$HCl), and 3-N, N, N-trimethylamino butyl ester iodide (3-TMABE2 iodide), were synthesized after preparation of the appropriate acid chloride following the procedure reported by Hussain et al [5]. The alcoholic ester, 17-N, N-dimethylamino butyl ester hydrochloride (17-DMABE$_2$HCl), was prepared by dissolving the acid chloride slowly in 10 mL N, N-dimethylformaride (DMF) while in an ice bath since the reaction is exothermic. 17ß-Estradiol was dissolved in methylene chloride, and the DMF solution of acid chloride was added dropwise to the solution of estradiol with stirring. The reaction mixture was refluxed gently for 45 minutes, then filtered. The filtrate was evaporated using a Buchi model rotavaprator (Westbury, New York), then redissolved in a small volume of 80

$CHCl_3$: 20 MeOH. The content of the mixture was separated and purified using a silica gel column. The solvent mixture was evaporated and the product redissolved in a small volume of methylene chloride, then hydrogen chloride gas was carefully bubbled through the solution with stirring. The ester hydrochloride was precipitated by adding enough diethyl ether to make the solution turbid and then the mixture was placed in a refrigerator (4% C) overnight. The final product was collected by solvent evaporation in a vacuum desiccator using a Precision Scientific model D75 pump (Chicago, IL) at room temperature and stored in a desiccator until used.

NMR spectra, mass spectrum, high-performance liquid chromatography (HPLC), melting point, and elemental analysis confirmed the structure and the purity of the esters.

Analytical Procedures

To determine physicochemical properties (ie, chemical stability, solubility, etc), as well as in-vitro enzymatic studies, HPLC chromatographic analysis was carried out in a system consisting of Applied Biosystems Solvents Delivery System 400 (Foster City, LA), Spectroflow 783 Absorbance Detector, Kratos Analytical Instruments (Westwood, NJ), 3390A Hewlett-Packard integrator (Palo Alto, CA), Waters Nova-Pack C8 column (3.9 mL × 150 mL) (Milford, CA), and C18 column ALTEX Ultrasphere (4.6 mL × 25 cm). The mobile phase consisted of 0.01M acetate buffer at pH 4 and acetonitrile. The acetonitrile portion was adjusted according to the ester. For (3-$DMABE_2HCl$) ester, the fraction of acetonitrile was 75%. For other esters, the fraction of acetonitrile was 50%. The flow rate was set at 1.0 mL/min. The UV wavelength was set at 254 nm for the phenolic esters and 280 nm for the alcoholic ester. The limit of quantification was 10 µg/mL.

For the in vivo studies, the system consisted of a radioimmunoassay procedure kit (Coat-A-Count) purchased from Diagnostic Products Corporation (Los Angeles, CA). The kit contains less than 6 microcuries of radioactive ^{125}I-estradiol. It is equipped with human serum-based calibrators, polypropylene tubes coated with rabbit antibodies to estradiol, and iodinated synthetic estradiol in liquid form. ^{129}I-labeled estradiol competes with estradiol in the tested samples for antibody sites. After 3 hours of room temperature incubation, separation of bound from free is achieved by decanting. The tube is then counted in a gamma counter, the counts being inversely related to the amount of estradiol present in the sample. The quantity of estradiol in the sample was determined by comparing the counts to a calibration curve. The antiserum is highly specific for estradiol with very low cross reactivity to other compounds that might be present in tested samples.

Stability of the Ester Prodrugs in Aqueous Buffers

The reactions were initiated by preparing 0.2 mg/mL solutions of the ester prodrug in phosphate buffers at the desired pH and concentration. The solutions were kept in screw-capped culture tubes at 37%C;. At appropriate time intervals, samples were taken and kept on ice until analysis.

In Vitro Enzymatic Hydrolysis Studies

Rat brain homogenate was obtained by homogenizing 1 part of whole rat brain tissue with 5 parts of saline using a tissue grinder. The studies were conducted by adding five 200-µL aliquot parts of rat plasma, brain homogenate to five 100-µL of a 0.05M phosphate buffer solution pH 6, containing 0.5 mg/mL of the appropriate ester and the samples incubated at 37ºC. The reactions were quenched at various times by adding 200 µL of acetonitrile. The samples were centrifuged for 2 minutes. The supernatant was filtered through a 0.45-µm filter and injected directly onto the HPLC.

In Vivo Studies

The nasal absorption of estradiol and the estradiol prodrugs was studied using an in vivo experimental technique described by Hussain et al [13,14]. All animal experiments adhered to the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) and were approved by the University of Kentucky Institutional Animal Care and Use Committee. Male Spargue-Dawley rats weighing 250-300 g were used.

All surgical procedures were performed under anesthesia (intraperitoneal injection of pentobarbital 40 mg/kg). The nasal cavity was isolated from the respiratory and gastrointestinal tracts. Blood samples were collected from a cannula inserted into the femoral artery. For intravenous administration, the jugular vein was cannulated for administering the dose. For nasal studies, the dose (100 µL) was administered into 1 nostril using a microsyringe. The 2 nostrils are connected, so the solution enters both cavities. CSF samples were obtained at the desired time by making an incision in the skin over the occipital bone and removing the first layer of muscle. Samples (75 µL) were then obtained by a cisternal puncture with a sharp-end needle. Sampling was stopped immediately on observing any trace of blood and the last 25 µL of each sample was discarded to prevent blood contamination. The animal was then killed.

RESULTS AND DISCUSSION

Physicochemical Properties

Table 1 lists the physicochemical properties of the prodrugs (as hydrochloride salts) in comparison to 17ß-estradiol. As evident from the data, the prodrugs are significantly more soluble than 17ß-estradiol.

Chemical Stability

The hydrolysis of all esters in phosphate buffer at 37°C and pH 7.4 followed first-order kinetics. The half-lives of hydrolysis for all the esters under the same condition are listed in Table 1. It is apparent that the alcoholic ester 17-$DMABE_2HCl$ is the most stable prodrug and that 3-TMABE2 iodide is more stable than 3-$DMABE_2HCl$. The difference in the rate of hydrolysis of 3-$TMABE_2$ iodide and 3-$DMABE_2HCl$ is due to the involvement of the protonated nitrogen to form a stable transition state of hydrolysis [15,16]. To determine the effect of pH and buffer concentration on the stability of the esters, the hydrolysis of the 3-$DMABE_2HCl$ and 17-$DMABE_2HCl$ esters were studied in 0.01M, 0.05M, and 0.10M phosphate buffer at 37ºC and at different pHs. Typical first-order behavior was observed. A plot of the observed rate constants for the hydrolysis of both esters against phosphate buffer concentration (Figures 2, 3) at different pHs indicated that the hydrolysis is subject to specific as well as general acid-base catalysis. Specific acid-base catalysis is indicated by the different intercepts, while general acid base catalysis is indicated by the different slopes.

The overall equation for the rate of decomposition of estradiol esters in phosphate buffer can be written as follows:

$$-d\,[\text{Ester}]/dt = k_{obs} \times [\text{Ester}] \quad (1)$$

where:

$$k_{obs} = k_{H+}[H^+] + k_{OH-}[OH^-] + k_{H2O} + k_{H2PO4^-}[H_2PO_4^-] + k_{HPO4^{-2}}[HPO_4^{-2}] \quad (2)$$

The k values represent the specific rate constants associated with the various catalytic species. Rearranging equation 2 and expressing the concentration of the buffer species in term of total buffer concentration and K_a the dissociation constant for $H_2PO_4^-$ equation 2 can be written as:

$$k_{obs} = k_0 + [k_{H2PO4^-}(H^+/H^+ + K_a) + k_{HPO4^{-2}}(K_a/H^+ + K_a)] * [\text{Buffer}]_T \quad (3)$$

where

$$k_0 = k_{H+}[H^+] + k_{OH-}[OH-] + k_{H2O} \quad (4)$$

Table 1- Physicochemical Properties of 17b-Estradiol, Its Prodrugs, and Half-lives for the In Vitro Hydrolysis of Estradiol Ester Prodrugs in 0.05M Phosphate Buffer pH 7.4 (m=0.5 with NaCl), Rat Plasma, Rat Brain Homogenate at 37˚C.

Compound	Molecular Weight	Melting Point (˚C)	Aqueous Solubility (mg/mL)	Buffer $t_{1/2}$	Plasma $t_{1/2}$ (min)	Brain $t_{1/2}$ (min)
Estradiol	272.4	137-179	0.008			
3-DMABE$_2$HCl	421.9	200-203	0.80	5.6 min	0.83	1.16
3-DEAPE$_2$HCl	435.9	195-197	20.0*	30.1 min	---	---
3-TMABE$_2$iodide	527.3	229-232	3.0	34.65 hr	---	---
17-DMABE$_2$HCl	421.9	249-252	0.90	84.5 hr	1.92	2.28

Aqueous solubility was measured in 0.05M acetate buffer at pH 4 and 20˚C.

*Micellar solubility.

The overall hydrolytic rate constant in the absence of buffer is represented by k_0. According to equation 3, a plot of k_{obs} versus total buffer concentration $[buffer]_T$ results in a straight line with:

$$\text{Slope} = k_{H2PO4^-}(H^+/H^+ + K_a) + k_{HPO4^{-2}}(K_a/H^+ + K_a)$$

$$\text{Intercept} = k_0$$

From the linear regression equations of the 3 plots in Figure 2 we can calculate the specific rate constants associated with each species. The values for the specific rate constants for the hydrolysis of 3-DMABE$_2$HCl were:

$k_{H^+} = 5.17\ hr^{-1}\ M^{-1}$, $k_{OH^-} = 8.44*10^6\ hr^{-1}\ M^{-1}$, $k_{H2O} = 1.5*10^{-3}\ hr^{-1}$, $k_{H2PO4^-} = 0.09\ hr^{-1}\ M^{-1}$, $k_{HPO4^{-2}} = 6.65\ hr^{-1}\ M^{-1}$

While the values for the specific rate constants for the hydrolysis of 17-DMABE$_2$HCl were:

$k_{H^+} = 5.73\ hr^{-1}\ M^{-1}$, $k_{OH^-} = 1.2*10^4\ hr^{-1}\ M^{-1}$, $k_{H2O} = 1.98*10^{-3}\ hr^{-1}$, $k_{H2PO4^-} = 4.8*10^{-3}\ hr^{-1}\ M^{-1}$, $k_{HPO4^{-2}} = 1.03\ hr^{-1}\ M^{-1}$

The data would suggest that the rate of degradation of both esters in the neutral pH range is determined by the concentration of the hydroxide ion and the buffer species. Based on the magnitude of the rate constants, it is evident that the stability of both

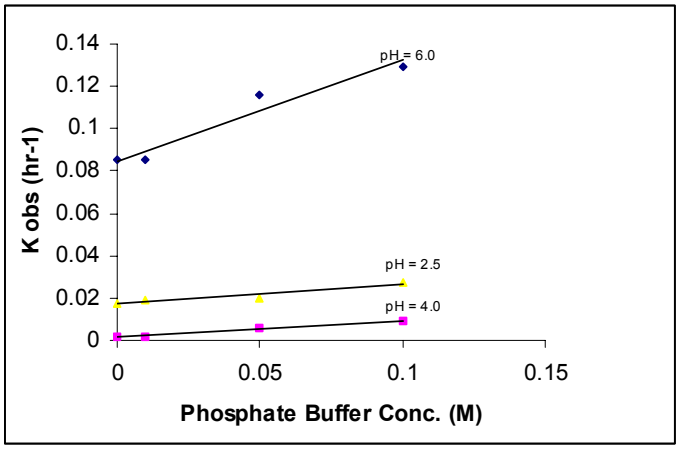


Figure 2 - Effect of buffer concentrations on the degradation rate constants of 3-DMABE$_2$HCl.

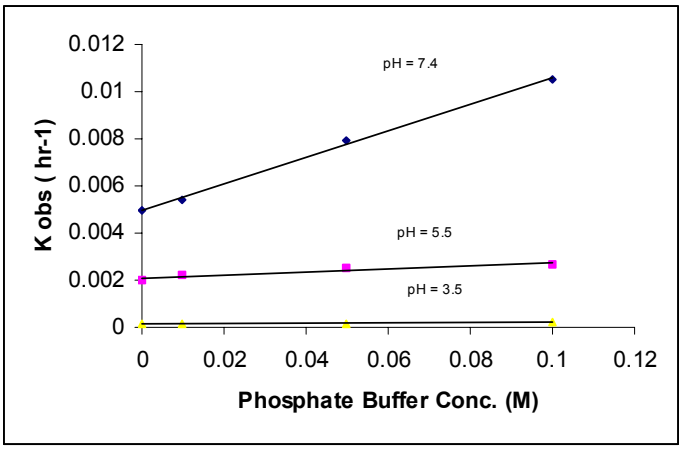


Figure 3 - Effect of buffer concentrations on the degradation rate constants of 3-DMABE$_2$HCl.

esters is apparently influenced by the pH of the solution and that at slightly acidic pHs (pH 3-5), 17-DMABE$_2$HCl prodrug would have sufficient shelf life to be formulated in a solution dosage form. For example, a pharmaceutical nasal spray solution of the prodrug at pH 4 would have a shelf life of approximately 19 months at 25ºC. Most nasal spray solutions are formulated in the pH range 3-4, and these solutions are known to cause no irritation to the nasal mucosa, especially if a low buffer concentration was used.

Enzymatic Stability

To verify whether these prodrugs can generate estradiol enzymatically in biological fluids, in vitro rates of hydrolysis of 3-DMABE$_2$HCl and 17-DMABE$_2$HCl were determined in plasma and brain homogenate. The hydrolysis of the prodrugs in rat biological fluids followed first-order kinetics; the rate of generation of estradiol in rat plasma was very rapid (Table 1). The generation of estradiol from 3-DMABE$_2$HCl and 17-DMABE$_2$HCl in rat brain homogenate, however, was much slower ($t_{1/2}$ = 1.16, 2.28 minutes) respectively.

In Vivo Studies with 17b-Estradiol and Its Prodrugs

Because the estradiol esters are converted to estradiol very rapidly in rat plasma, analysis of estradiol in the plasma following the nasal administration of the prodrugs should accurately reflect the absorption profiles of these esters. Figures 4 and 5 show plasma estradiol levels after nasal and intravenous administration of 3-DMABE$_2$HCl and 17-DMABE$_2$HCl prodrug esters at 0.1 mg/kg estradiol equivalent dose.

The area under the curve after nasal administration was calculated by using the MicroMath PK analyst Microsoft computer program (Redmond, WA). The bioavailability following nasal administration was obtained by comparing the area under the curves after intravenous and nasal administrations and was found to be about 103% and 78.5% respectively.

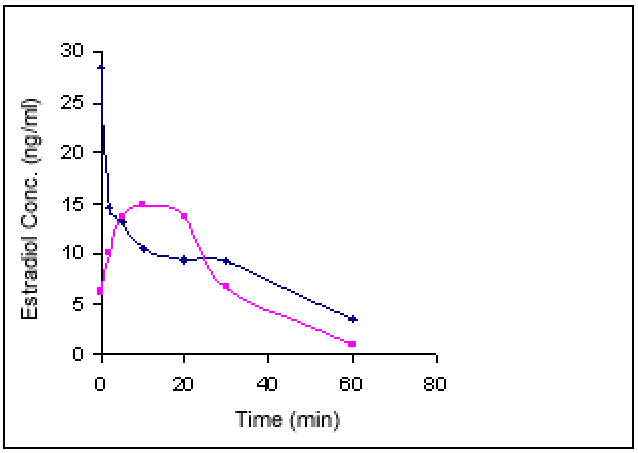


Figure 4 - Plasma estradiol levels following nasal and intravenous administrations of 3-DMABE$_2$HCl at 0.1 mg/kg estradiol equivalent dose.

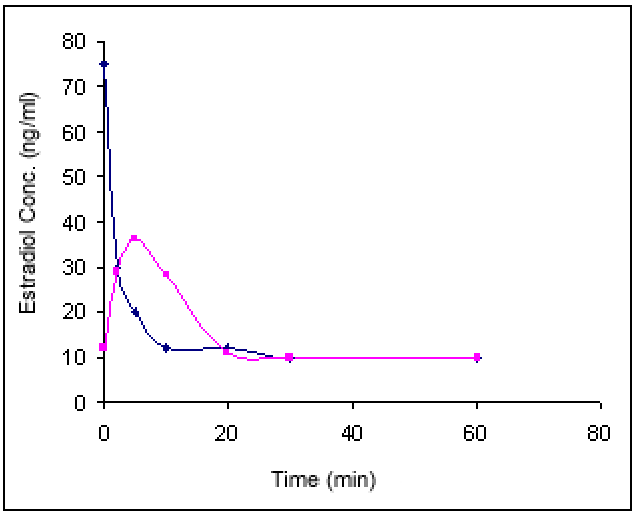


Figure 5 - Plasma estradiol levels following nasal and intravenous administrations of 17-DMABE$_2$HCl at 0.1 mg/kg estradiol equivalent dose.

CSF Estradiol Levels Following Nasal and Intravenous Administration of the Prodrugs

The CSF concentrations of estradiol following the intravenous and nasal administrations of 3-DMABE$_2$HCl and 17-DMABE$_2$HCl at 0.1 mg/kg estradiol equivalent dose are shown in Table 2. It is evident that the CSF has a higher concentration of estradiol following nasal administration than following intravenous administration. These data

Table 2- Cerebrospinal Fluid (CSF) Concentrations and Ratio of Estradiol Following the Intravenous and Nasal Administrations of Estradiol Ester Prodrugs*.

Prodrug	CSF levels after intravenous (ng/mL)	CSF levels after intranasal (ng/mL)	CSF ratio (in/iv)
3-DMABE$_2$HCl	7.53 ± 4.8	66.5 ± 8.1	8.8 ± 1.7
17-DMABE$_2$HCl	6.49 ± 3.1	30.59 ± 4.04	4.7 ± 1.3

*CSF estradiol levels ± SD.

suggest that the drug can reach the CSF via a direct pathway through the nasal cavity.

CONCLUSIONS

Estradiol prodrugs show significantly more water solubility than 17ß-estradiol. The conversion of the prodrugs to estradiol in the plasma was very fast as is evident by the in vitro data.

The nasal administration of estradiol prodrugs resulted in rapid and complete absorption into the systemic circulation. Furthermore, the nasal administration of the ester prodrugs resulted in an improved CSF bioavalability compared to that achieved from an equivalent intravenous dose.

In view of the above, the alcoholic prodrug (17-DMABE$_2$HCl) was found to be chemically stable enough to be formulated as a nasal spray solution. Therefore, 17-DMABE$_2$HCl is a good candidate to assess its potential for use in the management of Alzheimer's disease in women.

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