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Pre-clinical investigation of the topical administration of phenserine: transdermal flux, cholinesterase inhibition and cognitive efficacy.

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AChE: acetylcholinesterase

APP: amyloid precursor protein

BChE: butyrylcholinesterase

CSF: cerebrospinal fluid

FAPG: fatty alcohol propylene glycol

HPE-101: (1-[2-(decylthio)ethyl] azacyclopentane-2-one)

LP: liquid paraffin

NFT: neurofibrillary tangle

PS: phenserine

PST: phenserine tartrate

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ABSTRACT

Phenserine (PS) was designed as a selective acetylcholinesterase (AChE) inhibitor, with a tartrate form (PST) for oral administration in mild to moderate Alzheimer's disease (AD). Recent phase 3 trials of PST in Europe, indicate that any clinically relevant activity of PST may be limited by its duration of action.   Like many oral drugs, bioavailability and plasma concentrations of PST are regulated by hepatic and gastro-intestinal first-pass effects. In order to minimize the kinetic limitations of first-pass metabolism, transdermal formulations of PS and PST (ointment/patch) were developed and characterized *in vitro* and *in vivo*.   Initial *in vitro* kinetic characterization of PS or PST formulations employed a diffusion cell chamber and skin samples isolated from hairless mice. Liquid paraffin and fatty alcohol/propylene glycol (FAPG) were found to be suitable vehicles for ointment formulation. Addition of a penetration enhancer, 1-[2-(decylthio)ethyl] azacyclopentane-2-one (HPE-101), improved stratum corneum permeability. Application of the optimal formulation of PS/HPE-101/FAPG to the shaved back of rats, resulted in significantly lowered plasma and brain AChE activities and improved cognitive performance in animals with scopolamine-induced cognitive impairment. These results suggest that the transdermal application of AChE inhibitors may represent an effective therapeutic strategy for AD. Particular benefits over oral therapies might include avoiding first-pass metabolic effects and improved dosing compliance.

INTRODUCTION

Accompanying the increased geriatric population of most industrialized countries is an upsurge in the prevalence of dementia, particularly Alzheimer's disease (AD). AD is characterized by progressive neuronal loss leading to cognitive decline and a plethora of accompanying psychiatric problems. Pathologically, AD is characterized by the presence of (i) senile plaques; extracellular deposits primarily composed of β-amyloid (Aβ) being derived from amyloid precursor protein (APP) (Sambamurti et al., 2002; Selkoe 2005), (ii) neurofibrillary tangles (NFTs) of phosphorylated tau protein (Tanzi, 2005), and (iii) cholinergic synaptic and neuronal loss, with associated brain atrophy (Whitehouse et al., 1982; Doucett et al.,1986).

Cholinesterase inhibitors (ChE-Is) attenuate the cholinergic deficit considered to underlie the dysfunctions in AD, and, to date, represent the most widely employed treatment strategy (Lahiri et al., 2004). Four CHE-Is are approved in the US (tacrine: *Cognex*, donepezil: *Aricept*, rivastigmine: *Exelon*, and galantamine: *Reminyl*). Whereas this drug class and the recently FDA approved NMDA-receptor antagonist, memantine (*Namenda*), are the only agents to have been consistently associated with improvements in cognitive function in AD (Leo et al., 2006). Such improvements are, unfortunately, generally small. This modest efficacy has provided impetus to develop a new generation of ChE-Is with activity beyond symptomatic benefits, and maximize the efficacy of current agents based on a more complete understanding of time- and concentration-dependent enzyme/inhibitor interactions. Our research in both areas has focused on

mechanisms that reduce levels of neurotoxic Aβ, in addition to cholinesterase inhibition.

We designed and developed the acetylcholinesterase (AChE) selective inhibitor, phenserine (PS) ((-)-phenylcarbamoyl eseroline) (Fig. 1), which possesses additional non-cholinergic actions to lower the rate of APP synthesis and thereby reduce Aβ levels (Greig et al., 2005; Shaw et al., 2001).   Clinical trials to date with phenserine and the other approved ChE-Is, have involved oral administration and water-soluble salt forms of the compounds, eg. PS tartrate (PST). The oral route is most often used to administer therapeutics to humans, as it is convenient, safe and inexpensive. However, oral administration has limitations associated with bioavailability loss through first-pass metabolic and transport effects in the intestinal wall and liver. To avoid these critical metabolic sites, alternative administration routes have been investigated.

Transdermal application provides a potential approach for efficient and effective administration.   Although the transdermal route is most often utilized in local treatment, there may be therapeutic advantages for systemic therapy that include ready maintenance of steady-state drug levels, amelioration of peak concentration effects, and a lack of hepatic first-pass metabolism and gastrointestinal transport effects. Moreover, and of particular relevance for treatment of dementia, transdermal application may help achieve dosing compliance. Transdermal delivery necessitates optimization of permeability by modulating the physicochemical characteristics of the therapeutic, such as solubility, diffusion and enzymatic stability (Okuyama et al., 1999). It has been suggested that preferred characteristics of a therapeutic for transdermal application might include a

molecular weight < 500 and a lipid/water partition coefficient (log P) value of

approximately 2.5 (Ozawa et al., 1988).

PS is a crystalline compound developed through optimizing the structure/AChE activity

relationships of hexahydropyrrolo[2,3b]indole carbamates (Greig et al., 2005a), the

backbone of the classic anticholinesterase and natural alkaloid, physostigmine. The

unsubstituted phenylcarbamate of eseroline, PS ($C_{20}H_{23}N_3O_2 \cdot C_4H_6O_6$) is used

biologically as a L (+)-tartrate salt to aid its aqueous solubility. The free base form of PS

has a M.W. of 337.4 and is lipophilic, with a log P value of 2.2. It has a high brain

penetration (brain/plasma ratio of 10:1), a moderately long duration of action in rodents

(half-life, $t^1/_2$, 8.25 h) and a preferential selectivity for AChE vs. butyrylcholinesterase

(BChE) of approximately 70-fold (Greig et al., 2000). Such characteristics have been

purported to make ChE-Is more tolerable in humans (Greig et al., 1995, 2005a) and,

furthermore, make PS suitable for transdermal administration.

Clinical studies with phenserine have indicated that it is well tolerated by the oral route

and dose-limited by classical cholinergically-mediated adverse events, primarily nausea

and vomiting (Greig et al., 2005a,b). The onset of AChE inhibition was rapid and

occurred shortly after oral administration, reaching a maximum at 1.5 to 2.0 h and then

declining relatively slowly, with a dose-dependent $t^1/_2$ of 5-11 h. However, extensive

metabolism occurred, with plasma drug levels falling rapidly beyond the Cmax which

occurred at 1.5 h post oral administration (Greig et al., 2005b). These findings indicated

that transdermal administration might provide pharmacokinetic advantages, both for

maintenance of AChE inhibition, and also plasma drug concentrations to optimize non-

cholinergic actions on lowering $A\beta$. Studies were therefore initiated to characterize the

suitability of PS for transdermal formulation.

METHODS

Materials. PS and PST (>99.9% optically and chirally pure) were synthesized as described previously (Yu et al., 2001).   HPE-101 was obtained from Hisamitsu Pharmaceutical Co. (Saga, Japan). Other chemicals were purchased from Wako Pure Chemical Ind. (Osaka, Japan) and Sigma (St Louis, MO, USA).

Drug Formulation. Solids of PS or PST were mixed with various ointment vehicles, as listed in Table 1. Fatty alcohol/ propylene glycol (FAPG) ointment was prepared with various alcohols and propylene glycol.   Briefly, stearyl alcohol (9.5g), cetyl alcohol (8.0g) and 1-docosanol (12.0g) were melted at 85°C and stirred into propylene glycol (70.5g) at 90°C.   During subsequent cooling, the agents were continuously mixed for 20 min to form a paste (FAPG ointment). The permeability enhancer HPE-101 was added to some formulations, with further mixing, at concentrations of 1-5% w/w. Finely ground PS or PST was then blended into the FAPG ointment.   The final concentration of PS or PST within vehicle was 1% w/w for *in vitro* studies and 10% or 20% w/w for *in vivo* studies.

In Vitro **Transdermal PS Permeability.** A diffusion cell chamber (flow-through cell, Fig.1) was utilized (Addicks et al., 1987; Sclafani et al., 1993) to assess the availability of PS from vehicle, and subsequent transdermal permeability.   Since the stratum corneum has been characterized as a major barrier to transdermal permeability (Sheuplein and Blank, 1973; Madisonet al., 1987; Smith et al., 1982) and barrier integrity may be variably affected by age (Ghadially et al., 1995), permeabilities were investigated through both intact and tape-stripped skin. Intact skin or tape-stripped skin was isolated from the

back of hairless mice and placed between the donor and the acceptor phase of the flow-through cell allowing a permeation surface area of $1cm^2$.   Stripped skin without the stratum corneum was prepared by stripping with Scotch tape (Scotch[TM] 845) over some 30 occasions.   Formulated ointment was placed on the skin within the donor phase. Thereafter, saline (37º C) was circulated within the acceptor phase at a rate of 5ml/hr. Serial 2ml samples were collected from the acceptor phase at specified times.   PS concentrations were quantified by HPLC under the following conditions, column: octadecyl silica gel reversed phase (C18, 5 µm, 4mm i.d. x 150mm); mobile phase: methanol water (9:1 v/v); flow rate: 0.2 ml/min; detection: UV at 246nm.   The cumulative permeated amount of PS (Q, $\mu g/cm^2$) and steady state flux rate (Flux; $\mu g/h/cm^2$) were determined from PS concentrations of each fraction (i) as follows;

$$Q(\mathbf{ti}) = Q(\mathbf{ti\text{-}1}) + (C(\mathbf{ti}) \times V(\mathbf{ti})\,/A)$$

$$Flux(\mathbf{ti}) = (Q(\mathbf{ti}) - Q(\mathbf{ti\text{-}1}))/((ti) - (ti\text{-}1))$$

i : number of fractions

ti : sampling time (hr)

Q($\mathbf{ti}$) : cumulative permeated amount of PS at ti ($\mu g/cm^2$)

C($\mathbf{ti}$) : concentration of PS in sample at ti ($\mu g/ml$)

V($\mathbf{ti}$) : sampling volume between ti-1 and ti (ml)

A : area available for permeability (cm^2).

Flux(ti) : flux at ti (hr)

Further *in vitro* studies investigated the effects of modulating the surface area available for transfer of the PS in the diffusion cell. This was necessary prior to undertaking *in vivo* efficacy studies to ensure that sufficient transdermal PS flux occurred to sustain systemic levels within the previously established therapeutic range for the rat.

***In Vivo* transdermal PS permeability, and efficacy on cognitive performance and AChE activity.** Animals were lightly anesthetized (fluorothane) and carefully shaved to the skin in the mid-riff region of their back, facilitating the attachment of transdermal patches. FAPG ointments (+/- PS) were applied to the shaved backs of adult (4 month old) male Fischer-344 rats (Charles River) using two pads (250mg ointment per $4cm^2$ surface area pad; 500mg total ointment in $8cm^2$ surface area for each animal). Ointments were formulated with PS at 10% (w/w) with and without HPE-101 (5% w/w). Plasma AChE activity was then monitored in tail-blood, plasma samples for 8 hr. At the end of experiment, animals were killed by fluorothane overdose. Brain and plasma samples were collected and immediately frozen at -70ºC for later determination of AChE activity by the Ellman method (Ellman et al., 1961). Plasma was additionally analysed for biochemical markers of hepatic, renal and muscle function (aspartate aminotransferase, alanine aminotransferase, leucine aminopeptidase, leucine aminopeptidase, lactate dehydrogenase, alkaline phosphatase, albumin, creatine kinase, total protein, serum creatinine, blood urea nitrogen, triglycerides, total and free cholesterol, and phospholipids).

Adult (4 month old) male Fischer-344 rats (Harlan, Indianapolis, IN) were also employed
to assess the *in vivo* efficacy of transdermal PS on cognitive performance and
cholinesterase activities in plasma and brain (cerebral cortex). Employing previously
described protocols, we investigated the ability of transdermal PS to correct learning
impairment induced pharmacologically with scopolamine, and assessed in a 14-unit T-
maze (Ingram 1988). Animals were first trained to criterion in one-way active avoidance
in a straight runway. Thereafter, trials which involved negotiation of five maze segments
to avoid footshock, were conducted in a 14-unit T-maze. On Day 1, each rat was trained
in a straight runway to move from a start box to a goal box (~ 1m) within 10 seconds
while avoiding a mild footshock (0.8 mA). Training success was indicated by 13 out of
15 correct avoidances (maximum trials=30). Training assured that the animals had
learned footshock avoidance prior to the maze learning trials.

Drug Treatment: On Day 2 animals were lightly anesthetized (fluorothane) and carefully
shaved to the skin in the mid-riff region of their back, facilitating the attachment of
transdermal patches. Thereafter, animals were randomly assigned to one of three
treatment group; (i) control: physiologic saline (i.p. 1ml/kg body weight) + transdermal
vehicle ointment, (ii) scopolamine treatment: scopolamine (0.75mg/kg, i.p., in
physiologic saline;1ml/kg) + transdermal vehicle ointment, and (iii) scopolamine and PS
co-treatment: scopolamine (0.75mg/kg, i.p., in physiologic saline;1ml/kg) + transdermal
PS ointment. Two adhesive bandages (3.5 x 3.5cm with a 2.0 x 2.0cm gauze pad),
containing 250mg of PS ointment or vehicle, were applied to the shaved area on the back
of each rat. Rats in the PS treatment group received 50mg of PS formulated into vehicle

ointment, as described above. Three hours prior to maze testing on Day 3, the adhesive

bandages were removed and replaced with bandages containing fresh applications of PS

ointment or vehicle.   Following transfer to the testing room, and 30 minutes prior to

testing in a 14-unit T-maze, i.p. injections of either physiologic saline or scopolamine

were administered.

Cognitive Performance and Cholinesterase Inhibition: All rats performed 20 trials in a

14-unit T-maze with an inter-trial interval of 2 min. The performance measure was the

number of errors committed on each trial.   Immediately following the final maze run,

animals were killed by fluorothane overdose. Plasma and brain samples were collected

and immediately frozen at -70ºC. Cholinesterase levels were later determined by the

Ellman method (Ellman et al., 1961).

Data analysis.

All data are reported as means ± SEM of at least 4 trials or animals. Statistical

significance was determined by ANOVA with post-hoc LSD, Dunnet's and unpaired t-

tests as appropriate**.**

RESULTS

Permeability of PS and PST *in vitro.*

Phenserine was utilized as free base (PS) and tartrate (PST) forms in studies to identify

the most suitable ointment vehicle (Table 1) and active agent for a transdermal

formulation. The actions of the penetration enhancer HPE-101 were also assessed. PS or

PST (1% w/w) was formulated in various vehicles containing 0-5% (w/w) HPE-101.

Figure 2 shows the permeation profiles of PS from various vehicles containing PS or PST

through intact skin from the hairless mouse into receptor phase. Only the LP and FAPG

vehicles allowed sufficient permeation of PS through the intact dermal barrier.

Cumulative amounts of PS from the PS-containing vehicle recovered during the 8 hr of

the study were greater than those from the PST-containing vehicle, although the flux of

PS for the PST-containing vehicle began earlier than that for the PS-containing vehicle.

Subsequent studies focused on PS formulated within LP and FAPG vehicles.

Figure 3 and Table 2 present data regarding the effects of the penetration enhancer HPE-

101 (0-5% w/w) on total flux and steady state flux rates of PS, from LP and FAPG

formulations, through intact skin. Permeabilities of PS were markedly improved by the

addition of HPE-101. Maximum benefits of HPE-101 addition were observed between 3-

5% w/w HPE-101 concentrations.

As the stratum corneum is an impediment to transdermal permeability in intact skin and

its integrity may be reduced by age, we also examined PS permeability from formulated

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LP and FAPG ointments using stripped skin from the hairless mouse. Figure 4 shows

the permeation profiles of PS from these vehicles through both intact and stripped skin.

Cumulative flux of PS was increased in stripped versus intact skin, by 2.9- and 1.8-fold

for LP and FAPG ointments, respectively.

In order to study the *in vivo* efficacy of transdermal PS, it was necessary to ensure

sufficient total transdermal flux of PS to achieve systemic levels within the therapeutic

window for PS in the rat. It has been reported that the therapeutic window for PS in rat

plasma for maintenance of brain AChE inhibition is in the region of 50-100nM (Greig et

al., 2000), which is in accord with its brain AChE IC_{50} value of 36nM (Greig et al.,

2005a). To reach this therapeutic concentration with transdermal application, the optimal

flux of PS was calculated using various pharmacokinetics parameters assessed in

previous studies employing intravenous administration of PS. The optimal flux rate was

calculated using: Flux = ke x Vd x Css / A. Where ke, Vd, Css and A are rate constants

for elimination, distribution volume, systemic PS level at steady state, and area available

for transfer, respectively.   Based on approximately 200g body weight, ke and Vd were

calculated to be $3.3hr^{-1}$ and 1103.2ml. To achieve the necessary systemic PS level, a flux

rate of 60-120µg/hr was calculated to be necessary. Data presented above (Table 2) show

that the experimental fluxes of PS in the series of experiments employing a $1cm^2$ area of

intact skin for permeation within the diffusion cell, were approximately 5-fold lower than

that required to achieve systemic levels of PS within the therapeutic range. However, the

flux rate could theoretically be increased by providing a greater surface area for

absorption.

Having determined the need for a flux of 60-120μg/hr in order to achieve appropriate

systemic PS levels, we investigated PS fluxes using a diffusion cell with 5cm^2 area of

intact skin for permeation to compare with 1cm^2 data reported above. Formulations also

contained 5% (w/w) HPE-101. Figure 5 shows permeation profiles of PS from LP and

FAPG vehicles for 1cm^2 and 5cm^2 diffusion cell areas employing intact hairless mouse

skin.   The 5-fold increase in available diffusion area resulted in 4.3- and 8.2-fold

increases in total accumulated transdermal flux over a 12 hour period, for LP and FAPG

formulations, respectively.   Figure 6 shows that fluxes of PS from both vehicles entered

the range of 60-120μg/hr with 5cm^2 diffusion areas of intact skin, in the presence of 5%

(w/w) HPE-101.   It should be noted that the time necessary to reach the sufficient flux of

PS from the FAPG vehicle was shorter than that from the LP vehicle. This would be

theoretically sufficient to support systemic PS levels within the therapeutic dose window.

The in vitro findings suggest that LP and FAPG vehicles have almost the same

performance for delivering PS through hairless mouse skin and into the systemic

circulation.   To develop a final dosage form for transdermal PS, the LP vehicle requires

an additional pharmaceutical processing.   In contrast, the FAPG vehicle enables us to

prepare the ointment of PS without any processing.   Based on the above consideration,

we decided on the continued use of FAPG vehicle for in vivo studies.

**In Vivo transdermal PS permeability and efficacy effects on cognitive performance
and AChE activity**

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FAPG vehicle application alone, with or without HPE-10, did not affect plasma AChE activity as compared with untreated animals (data not presented).   As shown in Figure. 7, plasma AChE activity was significantly reduced by 30% following treatment with PS ointment containing HPE-101 (5%, w/w), versus controls receiving vehicle ointment alone. Concomitant inhibition of AChE activity in brain at 8 hr was 60% (p<0.05) following treatment with PS ointment containing HPE-101 (5%, w/w), versus controls. PS ointment without HPE-101, resulted in reductions in plasma AChE activity versus controls that did not achieve statistical significance. Plasma levels of biochemical markers of hepatic, renal and muscle function assessed at 1 and 8 hr, remained within their biological range for both PS treatments and controls (data not shown).

Figure 8 shows the effects of transdermal PS on cognitive impairment (errors in trial negotiation) induced with scopolamine.   Data indicate mean errors per block, across 4 blocks of five trials performed sequentially (Figure 8A) and mean errors per block, across the total 20 trials for each group (Figure 8B).   One-way ANOVA with repeated measures (4 blocks of 5 trials) yielded a main effect of drug treatment, $F_{(2,19)} = 51.77$, $p < 0.001$, and blocks of trials, $F_{(3, 57)} = 81.40$, $p < 0.001$. An interaction of drug treatment and blocks of trials was also observed, $F_{(6, 57)} = 4.38$, $p < 0.001$.   Post-hoc comparisons (LSD test; $p < 0.05$) of the mean errors per trial for each group were performed to determine the locus of the main effect. These analyses indicated that in scopolamine-treated animals significantly more errors were observed for all blocks as compared with the control group ($p < 0.05$).   PS treatment significantly decreased the number of errors induced by scopolamine, at blocks 3 and 4 ($p < 0.05$).   Furthermore, at block 4, the error

score in the PS group was not statistically different from that of the control group.

Across all 20 trials, PS treatment significantly reduced cognitive errors induced by

scopolamine, thereby improving overall maze performance (Fig. 8B, p < 0.01; Fisher,

LSD test).

Plasma and brain (cortex) AChE and BChE activities from the maze-tested animals are

shown in Table 3. Scopolamine treatment did not affect cholinesterase activities in

plasma or cortex, as compared with the control group and hence these groups were

combined. In contrast, co-treatment with scopolamine and transdermal PS, significantly

(p < 0.05) reduced AChE activities in plasma and brain by 68% and 66% respectively.

PS treatment lowered BChE activity in plasma by 18% (p < 0.05) and had no effect on

that in brain (p > 0.05).

DISCUSSION

Data from these investigations are supportive of the potential future use of PS as a transdermally applied therapeutic for AD and other conditions which feature a central cholinergic deficit.   Initial *in vitro* studies indicated the suitability of PS for transdermal application, as would be predicted from its physicochemical characteristics (Ozawa et al., 1988; Yu et al., 2001)

We examined PS and PST permeabilities through both intact skin and tape-stripped skin isolated from hairless mice and demonstrated the suitability of   LP and FAPG as vehicles in which the agent was formulated. For intact skin, *in vitro* total fluxes were greater for PS than PST, with release from FAPG being superior to that from LP. Furthermore, the time course profile of flux rates through intact skin (Figure 2B) clearly shows a superior, more predictable release of PS from vehicle as compared with PST. It would be expected that the free base (PS) would be readily released from vehicle as compared with the more water-soluble tartarte salt (PST). In the stripped skin studies, the formulation vehicle appeared to have little impact. It has been widely reported that the stratum corneum represents a major barrier to transdermal permeability (Sheuplein and Blank, 1973; Madison et al., 1987; Smith et al., 1982), and as expected, transdermal fluxes of PS were greater through stripped skin than intact skin. As the barrier integrity of the stratum corneum may be compromised with age and as AD is an age-associated disease, the former was assessed to provide an estimate of maximal permeability. However, the goal of developing a clinical application system, precluded the further investigation of a delivery system reliant on the absence of the stratum corneum. It has also been reported

that the use of an albumin solution in the receptor phase of the diffusion cell, to better

mimic sub-dermal physiologic conditions, may increase drug permeabilities by greater

than 5-fold, as compared with flux into pH adjusted saline solutions as in our studies

(Surber at al., 1991). Therefore, it may have been possible to achieve enhanced

permeabilities through the skin of the hairless mouse *in vitro* by modification of the

experimental conditions.

Several studies have shown that penetration enhancers are effective to improve fluxes and

subsequent bioavailability related to poor kinetic profiles of the transdermal

administration route (Aungst et al., 1986; Tsuzuki et al., 1988)   In these studies, we

examined the effectiveness of HPE-101, a well studied penetration enhancer (Yasuno et

al., 2001; Nakashima et al., 1996), to optimize the permeability of PS *in vitro*. HPE-101

appears to improve permeability through hydrophilic routes of the stratum corneum

without significant toxicity (Yano et al., 1992; Yano et al., 1993). Data indicated that the

optimal concentration of HPE-101 to increase PS flux, was in the range 3-5% w/w.

Using the *in vitro* permeability findings as a basis for the design of *in vivo* efficacy

studies, we modulated the area available for transdermal flux in order that the *in vitro*

model predicted the achievement of systemic, *in vivo* concentrations of PS within the

calculated therapeutic window for efficacy in AChE inhibition. This optimization was

also necessitated by reports of significant reductions of *in vivo* absorption rate (Flux) in

rats and humans compared with *in vitro* permeability data from hairless mice (Tregear,

1966; Iwasaki et al., 1999). Hence a diffusion area of $5cm^2$, was employed during the *in*

vivo permeability and efficacy studies performed in adult Fisher-344 rats.

The *in vivo* studies employed PS ointments with and without HPE-101, formulated in

FAPG. Statistically significant reductions (30% decrease at 8 hrs) in plasma AChE

activity were observed with PS (10% w/w) in the presence of HPE-101 (5% w/w). In the

absence of HPE-101, plasma AChE activities were also reduced, but less effective than

that with the penetration enhancer. It has been reported that intravenous administration

of PS (1.0 mg/kg) inhibits AChE activity in plasma and cerebrospinal fluid (CSF) of rats

by 60% and 90%, respectively.   This greater level of CSF inhibition is almost certainly

related to the observed brain:plasma partitioning of PS (10:1) (Greig et al., 2000).

Indeed, the AChE activity was dramatically reduced in brain samples (60% inhibition) as

compared with inhibition in plasma (30% inhibition) in our *in vivo* studies when HPE-

101 was included in the formulation. These results are suggestive of the requirement for

incorporation of a permeability enhancer in the ointment formulation in order to facilitate

PS movement through intact skin.   Furthermore, the levels of biochemical markers of

hepatic, renal and muscle function remained within their biological ranges, indicating that

administration of PS ointments, with and without HPE-101, were well tolerated. These

results suggest that selective reductions in plasma and brain AChE activity may be

achieved by the transdermal delivery of an agent such as PS, while avoiding some of the

pitfalls associated with oral administration, including hepatic toxicity and clearance as a

consequence of first-pass metabolism.

In order to assess the efficacy of transdermal PS treatment on cognition (or combined

higher brain functions/learning), we employed an accepted animal model of pharmacologically induced cognitive impairment (Ingram, 1988). In this model, animals trained to negotiate a 14-unit T-maze commit negotiation errors under the influence of the muscarinic antagonist, scopolamine. Scopolamine has been widely used to assist in the animal modeling of dementias involving a central cholinergic deficit (Ingram et al., 1994). Treatment with scopolamine induces rapid and significant reductions in rat brain cholinergic activity and causes learning impairment as assessed in the 14-unit T-maze (Iijima et al., 1993; Spangler et al., 1986).

In our studies, the transdermal administration of PS significantly reduced the number of cognitive errors induced by scopolamine. In a sequence of 20 trials (4 blocks x 5 trials), significant improvements in cognitive performance of the PS treated animals were observed beginning at trial 13. This compares favorably with our previous systemic treatment with PS (1.5 to 10 mg/kg, i.p.) using the same experimental paradigm, which resulted in significant effects occurring slightly earlier in the trials sequence; beginning at trial 8 (Lijima et al., 1993; Patel et al., 1998). The delay in onset of the PS effects in the transdermal model might be caused by the lag-time of PS release from the ointment and/or slow kinetics associated with transdermal absorption and systemic assimilation.

The improvements in maze performance were accompanied by selective inhibition of AChE activities in plasma and brain as measured in tissues collected at the end of the series of maze trials. BChE activities in plasma and brain were less affected by administration of transdermal PS. PS has previously been characterized as having a 70:1

preference for inhibition of AChE as compared to BChE (Greig et al., 2005a). It has been reported in AD patients, that improvements in cognition, and particularly of short term memory, correlate with the extent of plasma AChE inhibition, within the range of 0-50% inhibition (Becker et al., 1991; Greig et al., 1995). The data from this series of investigations clearly suggests that transdermal treatment with an appropriately formulated ointment of PS could have the potential to achieve levels of AChE inhibition which would provide similar clinical benefits.

In addition to a central cholinergic deficit which may be partly ameliorated by AChE inhibition with agents such as PS, AD is further characterized by the presence of central extracellular plaques, primarily composed of Aβ (Sambamurti et al., 2002). These deposits are considered to be intimately involved in the pathology of AD; although exact mechanisms remain to be elucidated (Selkoe, 2005). PS possesses additional non-cholinergic actions to reduce Aβ levels through lowering the rate of APP synthesis (Greig et al., 2005a; Shaw et al., 2001). It is likely therefore that PS delivered via the transdermal route, as in this series of studies, would exert beneficial effects on APP processing in addition to anticholinesterase activity. Further studies will be undertaken to elucidate these probable effects of transdermal PS on Aβ kinetics in this animal model. The transdermal application of agents such as PS may also provide real benefits with respect to treatment compliance. In a disorder such as AD where the ability to accept oral medications often progressively declines, the option of a therapeutic patch for drug delivery is especially attractive.

We conclude that these investigations demonstrate that PS may be a suitable candidate for transdermal administration; achieving transdermal flux of sufficient magnitude to support cognitive efficacy in an accepted animal model of cognitive impairment. Furthermore, the transdermal route avoids concerns of immediate hepatic toxicity and other deletarious consequences of first-pass metabolism. The slow release of active agent from ointment vehicle in a transdermal (patch) setting may also prove beneficial in sustaining the duration of action of PS, which has been suggested to be a limiting factor for clinical efficacy. These findings allow us to speculate that PS may therefore be formulated to provide clinical benefits when delivered transdermally that may help to reduce the cholinergic deficit of AD and possibly influence APP processing (Lahiri et al., 2006).

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. (A) Diffusion cell setup; (B) Structure of phenserine (PS)

Figure 2. I*n vitro* permeation profiles of PS from vehicles containing PS (1% w/w) or

PST (1% w/w), supplemented with HPE-101 (3% w/w), through 1 cm^2 intact skin into

saline at 37°C.

(A) Cumulative amounts of PS, (B) Flux of PS

●: PST from FAPG, O: PST from LP, ▲: PST from PG, Δ: PST from Hydrophilic

ointment, X: PST from hydrophilic petrolatum, ■: PS from FAPG, □: PS from LP, ◆:

PS from PG, ◊: PS from hydrophilic ointment, ✳: PS from hydrophilic petrolatum.

Each point represents mean ± SEM (N=4).

Figure 3. *In vitro* permeation profiles of PS from LP and FAPG vehicles containing PS

(1% w/w) supplemented with HPE-101 (0~5% w/w) through 1 cm^2 **intact** skin into saline

at 37°C.

●: from LP with HPE (5% w/w), O: from LP with HPE (3% w/w),

▲: from LP with HPE (1% w/w), Δ: from LP without HPE,

■: from FAPG with HPE (5% w/w), □: from FAPG with HPE (3% w/w),

◆: from FAPG with HPE (1% w/w), ◊: from FAPG without HPE.

Each point represents mean ± SEM (N = 4).

Figure 4. *In vitro* permeation profiles of PS from LP and FAPG vehicles containing PS

(1% w/w) supplemented with HPE-101 (3% w/w) through 1 cm^2 **intact** or **stripped** skin

into saline at 37°C.

■: PS from LP through stripped skin, □: PS from LP through intact skin,

●: PS from FAPG through stripped skin, O: PS from FAPG through intact skin.

Each point represents mean ± SEM (N=4).

Figure 5. *In vitro* permeation of PS from LP and FAPG vehicles containing PS (1% w/w)

supplemented with HPE-101 (5% w/w) through 1cm^2 or 5cm^2 **intact** skin into saline at

37°C.

●: from LP through 5 cm^2 intact skin, O: from LP through 1 cm^2 intact skin,

▲: from FAPG through 5 cm^2 intact skin, Δ: from FAPG through 1 cm^2 intact skin.

Each point represents mean ± SEM (N=4).

Figure 6. *In vitro* flux of PS from LP and FAPG vehicles containing PS (1% w/w)

supplemented with HPE-101 (5% w/w) through 1cm^2 or 5cm^2 **intact** skin into saline at

37°C.

●: from LP through 5 cm^2 intact skin, O: from LP through 1 cm^2 intact skin,

▲: from FAPG through 5 cm^2 intact skin, Δ: from FAPG through 1 cm^2 intact skin.

Each point represents mean ± SEM (N=4).

Figure 7. (Left) In vivo, rat plasma AChE activity during 8 hr transdermal administration

of FAPG ointment containing PS (10% w/w), supplemented with or without HPE-101

(5% w/w).

■: untreated control, O: FAPG ointment with PS (10% w/w), ●: FAPG ointment with PS

(10% w/w) and HPE-101 (5% w/w).

*P < 0.05, **P < 0.001 versus untreated control

Each point represents mean ± SEM (N=5).

(Right) Brain AChE activity at 8 hr in control rats and those administrated FAPG

ointment with PS (10% w/w) and HPE-101 (5% w/w) (p<0.05 PS treatment vs. control).

Figure 8. Effects of transdermal administration of FAPG ointments containing PS (20%

w/w), supplemented with HPE-101(5% w/w), on scopolamine-induced cognitive

impairment in rats. Errors assessed in 14-unit T-maze trials.

O: Control, Δ: Scopolamine, ■: Scopolamine + PS.

*P < 0.01 versus control, †P < 0.01 versus scopolamine group.

Each point represents mean ± SEM (N=7-8)

Table 1. Vehicles studied

Vehicle	Classification		
Liquid paraffin (LP)	Hydrophobic	Oleaginous base	Mineral
Hydrophilic ointment	Hydrophilic	Emulsion base	[o/w] emulsion
Hydrophilic petrolatum	Hydrophilic	Emulsion base	[w/o] emulsion
Polyethylene glycol	Hydrophilic	Water soluble base	
Fatty alcohol propylene glycol (FAPG)	Hydrophilic	Suspension	lyogel

Table 2. Steady state fluxes and cumulative amounts of *in vitro* transdermal permeation of PS (1% w/w in vehicle) across 1 cm^2 **intact** skin into saline at 37°C. Transdermal preparations were formulated with LP or FAPG vehicles and HPE-101 at 0-5% concentrations (w/w). (N = 4)

	Formulation		Steady state flux	Cumulative amount
Drug	Vehicle	HPE-101 (% w/w)	(μg/ hr/cm^2)	for 12 hr (μg)
PS	LP	0	0.6 ± 0.1	5.2 ± 41.2
		1	4.6 ± 0.7	37.0 ± 15.6
		3	17.4 ± 3.3	95.6 ± 14.3
		5	20.1 ± 0.3	182.9 ± 15.4
	FAPG	0	2.4 ± 0.2	16.6 ± 10.2
		1	11.0 ± 1.3	122.3 ± 19.4
		3	12.3 ± 1.5	117.3 ± 21.9
		5	8.2 ± 0.8	83.3 ± 13.2

Table 3. AChE and BChE activities in plasma and cerebral cortex of rats treated with and

without scopolamine (i.p.) and co-treated with PS ointment.

Treatments	Plasma (U/L ± SEM)		Cortex (U/g ± SEM)	
	AChE	BChE	AChE	BChE
Control	1754 ± 62	1006 ± 43	9687 ± 955	777 ± 60
(w & w/o scopolamine)	[14]	[14]	[14]	[14]
PS	$551 \pm 65**$	$822 \pm 32**$	$3242 \pm 385**$	783 ± 77
	[8]	[8]	[8]	[8]
Inhibition	68%	18%	66%	0%

Dunnett's t-test, **$P<0.01$, No. of animals shown in brackets

Fig. 1A

Donor phase

Hairless Mouse Skin

Receptor Phase

Outlet

Inlet

Stirrer
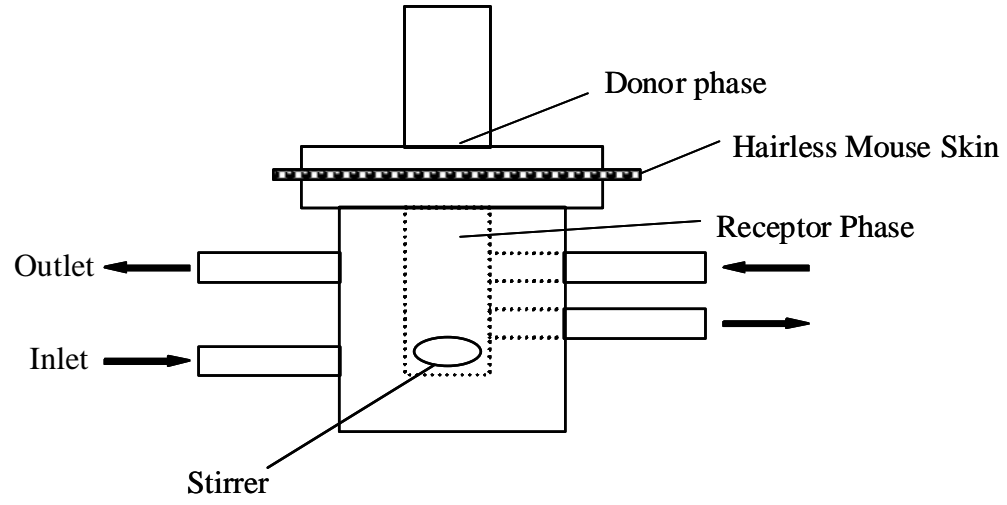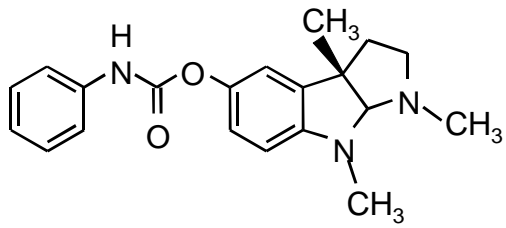
Fig. 1B

Fig. 2
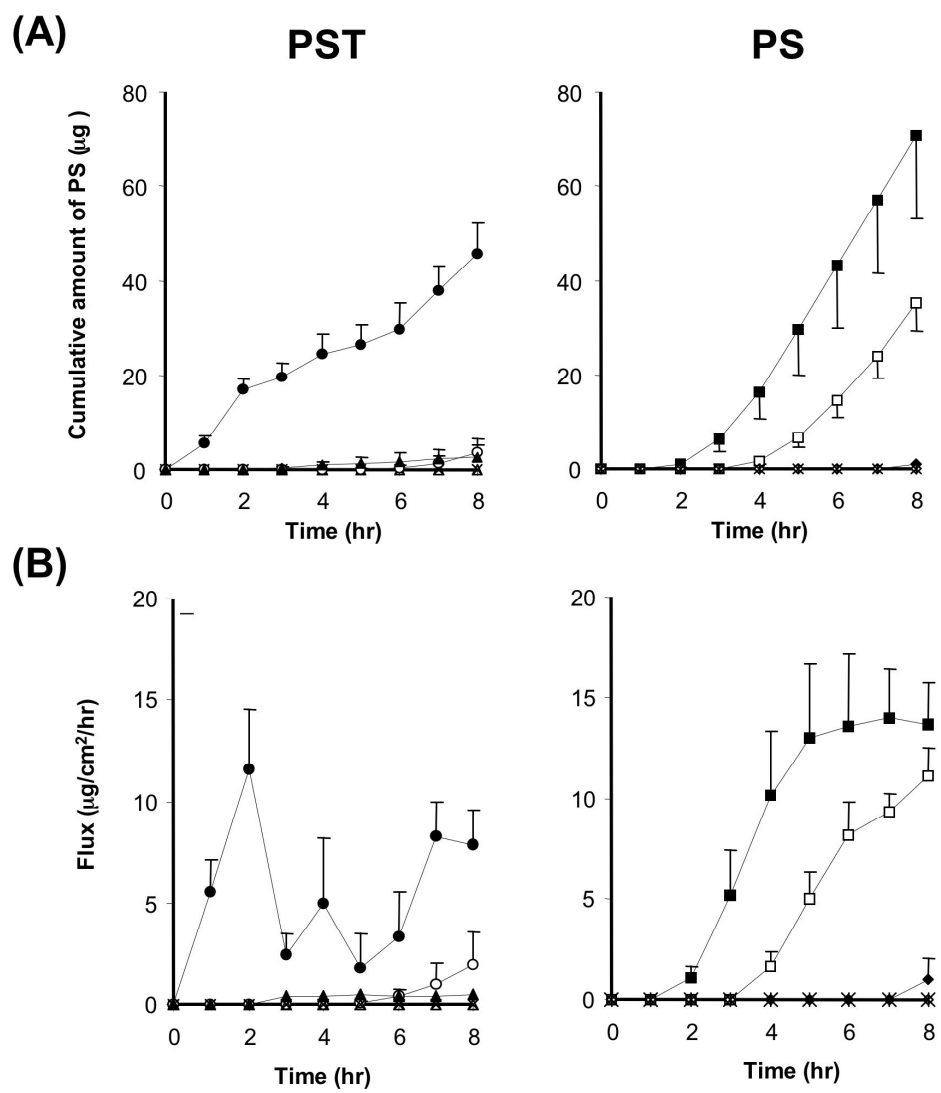
Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

(A)

(B)