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The Experimental Alzheimer's Disease Drug Posiphen [(+)-Phenserine] Lowers Amyloid-β Peptide Levels in Cell Culture and Mice

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ABSTRACT

Major characteristics of Alzheimer's disease (AD) are synaptic loss, cholinergic dysfunction, and abnormal protein depositions in the brain. The amyloid β-peptide (Aβ), a proteolytic fragment of amyloid β precursor protein (APP), aggregates to form neuritic plaques and has a causative role in AD. A present focus of AD research is to develop safe Aβ-lowering drugs. A selective acetylcholinesterase inhibitor, phenserine, in current human trials lowers both APP and Aβ. Phenserine is dose-limited in animals by its cholinergic actions; its cholinergically inactive enantiomer, posiphen (+)-[phenserine], was assessed. In cultured human neuroblastoma cells, posiphen, like phenserine, dose- and time-dependently lowered APP and Aβ levels by reducing the APP synthesis rate. This action translated to an in vivo system. Posiphen administration to mice (7.5–75 mg/kg daily, 21 consecutive days) significantly decreased levels of total APP (tissue mass-adjusted) in a dose-dependent manner. $A\beta_{40}$ and $A\beta_{42}$ levels were significantly lowered by posiphen ($\geq$15 mg/kg) compared with controls. The activities of α-, β-, and γ-secretases were assessed in the same brain samples, and β-secretase activity was significantly reduced. Posiphen, like phenserine, can lower Aβ via multiple mechanisms and represents an interesting drug candidate for AD treatment.

Alzheimer's disease (AD) is typified by progressive impairment in short-term memory and emotional disturbances that result from dysfunction and death of neurons in the hippocampus and associated regions of the limbic system and cerebral cortex. These aberrations are considered to result, in part, from microtubule-associated protein τ (τ) tangles and abnormal aggregates of cytoskeletal proteins (Cairns et al., 2004), oxidative stress, and the overproduction and accumulation of amyloid-β peptide (Aβ) in and surrounding neurons (Selkoe, 2005).

This 39- to 43-amino acid peptide (molecular mass ~4.1 kDa) is a core constituent of amyloid plaques and results from two catalytic cleavages of the larger integral membrane protein, amyloid-β precursor protein (APP; ~110–130 kDa), at the N terminus (β-secretase) and C terminus (γ-secretase) of Aβ (Sambamurti et al., 2002; Lahiri et al., 2003b; Selkoe, 2005). Significant evidence indicates that Aβ changes conformation from a physiological to a pathological, fibrillar peptide form, which not only induces local structural disruption of synapses and neurite breakage but also results in cell death due to perturbed calcium homeostasis and oxidative stress. In addition, soluble aggregates of Aβ or Aβ-derived diffusible ligands found in the brains of AD patients have been recently shown to target synapses (Gong et al., 2003) and play a role in inhibiting LTP (Walsh et al., 2002; LaFerla and Oddo, 2005). Conjointly, these studies point to the importance of Aβ in learning and memory, suggest a causative role of Aβ in AD pathophysiology, and thereby support its

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ABBREVIATIONS: AD, Alzheimer's disease; τ, microtubule-associated protein τ; Aβ, amyloid-β peptide; APP, Aβ precursor protein; CTF, C-terminal fragment; AChE, acetylcholinesterase; UTR, untranslated region; IL, interleukin; posiphen, (+)-phenserine; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; TCA, trichloroacetic acid; PCR, polymerase chain reaction; BChE, butyrylcholinesterase; FU, fluorescence unit(s).

reduction as a strategy for the treatment of AD (LaFerla and Oddo, 2005). Nevertheless, because toxic C-terminal fragments of APP can accumulate upon using γ-secretase inhibition, and APP-mediated neurodegeneration, beyond the accumulation of toxic Aβ forms, is known to occur in Down's syndrome models, reduction of APP and β-secretase derived C-terminal fragments (CTFβs) is an important goal for treatment of AD (Sambamurti et al., 2006). Moreover, presenilins have been shown to play an important role in γ-secretase activity, Aβ formation, and AD (Steiner, 2004; Wolfe, 2006).

A number of approaches are being investigated to lower brain Aβ levels and primarily focus on either increasing its clearance or reducing its synthesis. Regarding the former, monoclonal antibody-based vaccines directed against Aβ have led to plaque reductions in transgenic mice and improvements in cognition (Morgan and Gitter, 2004). Although a clinical trial using a vaccine directed against Aβ was halted, pathological data from patients within the trial support the notion that Aβ reduction may influence disease progression, as well as τ phosphorylation (Nicoll et al., 2003), a further pathological hallmark of AD (Selkoe, 2005). An action of Aβ on τ phosphorylation is also supported by immunization of triple-transgenic mice expressing the APP, τ, and presenilin-1 genes (LaFerla and Oddo, 2005).

A reduction in Aβ can similarly be induced by lowering its production through β- or γ-secretase inhibition, and inhibitors of these are in current AD preclinical and clinical assessment (Lahiri et al., 2003b; Vardy et al., 2005). To date, however, acetylcholinesterase (AChE) inhibitors remain the primary treatment strategy for mild to moderate AD subjects in the United States (Giacobini, 2003), to which the recently approved *N*-methyl-D-aspartate antagonist, memantine, has been added (Lleo et al., 2006). Although AChE inhibitors are widely considered to be symptomatic drugs that elevate brain acetylcholine levels and thereby augment learning and memory (Giacobini, 2003), specific inhibitors have been shown to affect APP processing in cell culture and to lower Aβ (Lahiri et al., 1994, 1998; Pakaski and Kasa, 2003; Racchi et al., 2004). Should such actions translate to animals at well tolerated doses and, in particular, to humans, such agents may prove valuable to slow AD progression (Giacobini, 2003; Lahiri et al., 2003b).

Our goal has been to investigate, design, and synthesize various classes of agents that can minimize cell dysfunction and death in neurodegenerative diseases (Greig et al., 2004, 2005). The regulation of APP expression represents an important untapped approach to AD treatment because it is the originator of not only Aβ but also other APP-derived toxic fragments, and is a product of both neurons and glia that is up-regulated in AD by endogenous and environmental factors, such as cytokines and heavy metals (Lahiri et al., 2003b). (−)-Phenserine (phenserine) (Fig. 1), a physostigmine analog and AChE inhibitor that has reached clinical assessment, lowers APP and Aβ levels in cell culture and animal models (Greig et al., 2005). This latter action is not mediated by cholinergic stimulation of alternative APP-processing pathways but represents the inhibition of APP mRNA translation mediated by signals in its 5′-untranslated region (UTR) (Shaw et al., 2001). This region of 5′-UTR APP mRNA is known to contain a translational enhancer that includes an iron-response element, interleukin (IL)-1) element (Rogers et al., 1999), and a transforming growth factor-β1-response element (Lahiri et al., 2003a) that are all elevated in AD brains (Venti et al., 2004). In addition, the iron-response element, IL-1 element, and a "CAGA" box participate in a structure similar to the "bulge-loop" structure of human immunodeficiency virus-1 transactivation-response element RNA (Maloney et al., 2004).

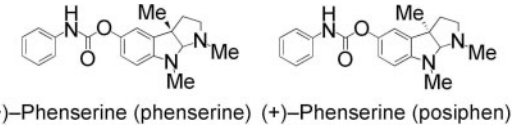

Fig. 1. Chemical structures of posiphen and phenserine. These are opposite, (+)- and (−)-, chirally pure enantiomers of each other [where a methyl (CH_3) moiety in the 3-position of the tricyclic ring either goes into the plane (hatched) or comes out of the plane, respectively, to represent mirror images of one another].

A potential concern for dual action drugs that inhibit AChE is that this latter action is generally dose-limiting. Therefore, we investigated whether posiphen (Fig. 1), a chiral isomer largely devoid of anticholinesterase activity (Yu et al., 1997; Greig et al., 2005), may effectively lower APP and Aβ levels and thereby represent an interesting AD drug candidate.

We report that posiphen, like phenserine, lowers levels of secreted and cellular APP and secreted Aβ (both short and long forms) in cultured neuronal cells. This translated to in vivo studies in mice, where brain levels of both APP and Aβ were dose-dependently lowered by posiphen over a wide dose range that was well tolerated and beyond that achievable with phenserine. Mechanisms underpinning the reduction in APP and Aβ involved reduction in the rate of synthesis of newly formed APP, assessed in cell culture. In addition, high doses of posiphen yielded mouse brain extracts showing significantly lower β-secretase activity. Posiphen treatment was also associated with greater cortical mass and greater protein content per wet cortical tissue mass, which may indicate a general neurotrophic function beyond its immediately measured effects on APP/Aβ levels and β-secretase activity. Posiphen hence represents an interesting AD clinical candidate as well as a lead compound in the development of β-secretase inhibitors.

Materials and Methods

Materials. Posiphen tartrate [(+)-phenserine tartrate] is the positive enantiomer of (−)-phenserine tartrate (Shaw et al., 2001). Both agents were synthesized as described previously (Yu et al., 1997) and were chemically and chirally >99.9% pure. Both the drugs were made either in phosphate-buffered saline for in vivo work or in the respective medium for cell culture studies.

Most chemicals were purchased from Sigma (St. Louis, MO) in the highest offered purity (analytic or molecular biology grade), unless stated otherwise. Primary antibodies against proteins, such as APP and β-actin, and secondary antibodies were purchased from Roche (Indianapolis, IN), Chemicon (Temecula, CA), or Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture. Human neuroblastoma cell lines SK-N-SH and SH-SY-5Y were obtained from the American Type Culture Collection (Manassas, VA), culture medium was from Mediatech, Inc. (Herndon, VA), and the fetal calf serum was from HyClone (Logan, UT).

Drug Treatment. SK-N-SH or SH-SY-5Y cells were cultured on 60- or 100-mm dishes at a concentration of 3 or 8×10^6 cells, respectively. Cells were allowed to grow in complete media (10% fetal calf serum, 2 mM glutamine in DMEM) for 2 days to reach 70%

confluence. Thereafter, spent media was removed and replaced with fresh media (2 ml of DMEM) containing 0 to 50 μM posiphen or phenserine. Cells were incubated at 37°C, 5% CO_2 for the specific times indicated.

Western Blot, Cell Viability, and Aβ in Cell Culture Experiments. Fifteen micrograms of protein from each sample was mixed with Laemmli buffer, boiled for 5 min at 100°C, and loaded onto a 10% SDS-polyacrylamide gel electrophoresis gel (Novex, San Diego, CA). The proteins separated at 150 V for 90 min, transferred to nitrocellulose membrane, and probed with 22C11 (2 μg/ml) antibody to APP. The monoclonal 22C11 antibodies recognize an N-terminal epitope of APP and were commercially obtained (Chemicon). The blots were incubated in secondary antibody, anti-mouse IgG- conjugated to horseradish peroxidase, for 30 min. Thereafter, samples were detected by chemiluminescence. Cell viability was assessed by measurement of lactate dehydrogenase levels (Lahiri et al., 1998). Levels of Aβ from conditioned medium were quantified by ELISA. Rabbit polyclonal antibody 3160 (Aβ_{40}) was used as a capture antibody for all species of Aβ (Aβ_{40} and Aβ_{42}), whereas monoclonal antibody 4G8 (Aβ_{17-25}) was used to detect Aβ levels, and the values were expressed as the mean of six independent assays.

Rate of APP Synthesis. After treatment (16 h) with and without posiphen and phenserine (10 μM), SH-SY-5Y human neuroblastoma cells (8×10^6 cells on 100-mm dish) were incubated in methionine and cysteine-free DMEM containing 4 mM glutamine for 1 h. Thereafter, the media was replaced with 2 ml of ^{35}S-labeled DMEM (100 μCi/ml) for 10 min. The labeled media was removed, cells were suspended in lysis buffer (20 mM HEPES, 2 mM EGTA, 50 mM β-glycophosphate, 1 mM sodium orthovanadate, 1% Triton X-100, and 10% glycerol) containing protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, leupeptin and soybean trypsin inhibitor). Isolated total proteins were quantified by BCA assay (Pierce, Rockford, IL), and equal amounts of protein (200 μg) were incubated with Ab2072 (Abcam Inc., Cambridge, MA) and protein resin A/G overnight at 4°C. Immunoprecipitated APP was eluted with 30 μl of elution buffer (10% β-mercaptoethanol). The samples were loaded onto 10% Tris-glycine gels, and the proteins were separated at 150 V for 90 min. The gels were fixed and dried at 80°C for 60 min. The dried gels were exposed onto a phosphorimaging screen (Perkin/Elmer, Wellesley, MA) overnight, and the APP signal was quantified on PhosphorImager. The levels of newly synthesized APP were normalized by ^{35}S-incorporated protein levels, which were trichloroacetic acid (TCA)-precipitable counts normalized by TCA-nonprecipitable counts. The assay was first optimized by titrating the antibody with given amounts of cell lysate. From this titration experiment, a saturation curve was established, and an optimal concentration of antibody was used at which total protein (200 μg of total protein) was at a subsaturating level. Using the optimal concentration of antibody, APP signals were detected between 100 and 120 kDa molecular mass, and no other signal was detected in any other range, indicating the specific binding of antibody to APP. In addition, during optimization, the compounds were shown to not interfere with antibody binding to APP.

APP mRNA. Total RNA was isolated from treated and untreated SH-SY-5Y cells using RNeasy (QIAGEN, Valencia, CA). Reagents for reverse transcription and specific PCR primers and probes for human APP and glyceraldehyde-3-phosphate dehydrogenase were procured from Applied Biosystems (Foster City, CA). Separately, various concentrations of extracted RNA from SH-SY-5Y cells were prepared to provide a relative standard curve, and quantitative reverse transcriptase-PCR was carried out with the PE Applied Biosystems Prism 7700 Sequence Detection System using the following parameters: one cycle of 2 min at 50°C and 10 min at 95°C and 40 cycles of 15 s at 95°C and 60 s at 60°C. The ABI Prism 7700 measured fluorescent emissions, which increased in direct proportion to the increase of amplified product continuously during the PCR amplification. Obtained data (threshold of cycle value) were calibrated to relative RNA (APP or glyceraldehyde-3-phosphate dehydrogenase) amounts using the relative standard curve with RNA extracts from SH-SH-5Y cells. RNA was isolated, and equal volumes were separated through a 1.2% agarose-formaldehyde gradient and probed for actin and APP using random primed ^{32}P probes. Signals of APP and actin mRNAs were quantified by phosphorimaging analysis on the Cyclone PhosphorImager (Perkin/Elmer).

Drug Treatment in the Animal. Adult male mice (C57BLk), 28- to 32-g weight, were obtained from Harlan Labs (Indianapolis, IN) and had free access to food and water. In accord with an approved the Institutional Animal Care and Use Committee protocol, 68 mice were weighed and injected once daily for 21 consecutive days i.p. with either saline (control) or posiphen, prepared in saline at a dose of 7.5, 15, 25, 35, 50, or 75 mg/kg. An additional 20 mice were administered phenserine (7.5 or 15 mg/kg in saline). Brains were rapidly harvested over ice between 90 and 120 min after the final injection and frozen to −80°C. The right cerebral hemisphere from each mouse was weighed after freezing. Analysis of hemisphere mass by median absolute deviation showed that two samples (one control and one posiphen, 7.5 mg/kg) were beyond 4 median absolute deviation from the rest of the set (both were low values) and were excluded from the analysis.

Preparation of Brain Extracts. Cerebral hemispheres were homogenized, and cell lysates containing whole protein were prepared according to the manufacturer's instructions for enzymatic assay kits (R&D Systems, Inc., Minneapolis, MN). In brief, the brain cortices were homogenized in 800 μl of low salt containing 1× extraction buffer (R&D kit) and incubated on ice for 10 min. The homogenates were then centrifuged at 13,000*g* for 15 min at 4°C. The protein concentration in each sample was estimated according to biuret-derived assay (Pierce). Levels of APP, Aβ_{40}, and Aβ_{42} were measured by Western immunoblotting and sandwich ELISA techniques, respectively, as described below. In addition, activities of α-, β-, and γ-secretases were measured from the same extracts, as described below.

Assay of Levels of Total APP in Mouse Cerebral Cortex Extracts. An equal-protein amount of brain extracts was loaded in each gel, 30 μg per lane per sample. Each gel contained eight treatments: control; phenserine, 7.5 mg/kg dose; and posiphen, 7.5, 15, 25, 35, 50, and 75 mg/kg doses. To minimize intragel variation, each gel contained three sets of eight treatment groups (set $n = 3$; total samples = 24). Additional gels/blots were performed to a total of 10 independent samples (except for control and 7.5 and 75 mg/kg posiphen, $n = 9$). Each blot was probed with 22C11 antibody as described previously (Lahiri et al., 1994). One representative photo of gel/blot was presented as shown on the top of the appropriate figures. Each blot was also probed with the β-actin antibody for normalization to transfer efficiency. The intensity of APP band signal was normalized with that of β-actin. Two sample "controls" were loaded in each gel/blot to test for blot to blot variation. Analysis of blots was done in two ways: blot wise ($n = 3$), to test for gel to gel variation; and combined blots ($n = 9$ or 10). Blot-wise analysis with $n = 3$ showed the same trend as the final analysis with $n = 9$ or 10 did. Results of the combined analysis are presented herein.

Assay of Levels of Aβ_{40} Peptides in Mouse Cerebral Cortex Extracts. Brain extracts used in this assay were subsamples of those tested in the APP assay. ELISA was performed to quantitatively assay levels of Aβ_{40} in different brain extract samples using an Immuno-Biological Laboratories kit that detects human as well as rodent Aβ (27713; Immuno-Biological Laboratories Company, Gunma, Japan), as described previously (Lahiri et al., 2004; Basha et al., 2005). The number of samples tested were control and all drug treatments, $n = 9$, except for 75 mg posiphen, wherein $n = 8$. Results from all experiments were adjusted to brain tissue mass and analyzed for statistical significance.

Assay of Levels of Aβ_{42} Peptides in Mouse Cerebral Cortex Extracts. Brain extracts used in this assay were the ones that were previously tested in APP and Aβ_{40} assays. A sensitive sandwich ELISA was performed to quantitatively assay levels of Aβ_{42} in dif-

ferent brain extracts samples under the same condition as described previously (Lahiri et al., 2004; Basha et al., 2005). The procedure is otherwise as described for measuring $A\beta_{40}$ peptide levels.

Assay of α-, β-, and γ-Secretase Activity. The activity levels of α-, β-, and γ-secretases were assayed using secretase-specific peptides conjugated to the fluorescent reporter molecules EDANS and DABCYL (R&D Systems, Inc.). The assays were set up in sterile 96-well plates using the tissue lysates prepared as above and reagents provided in kits. The lysates were incubated in the dark for 2 h at 37°C along with 50 μl of 2× reaction buffer and 5 μl of substrate. Specific kits were used for assessing α-, β-, and γ-secretase activity. After incubation, the plates were analyzed for fluorescence at 355/500 nm (excitation/emission).

Cholinesterase Measurements. The concentration of posiphen and phenserine required to inhibit 50% enzyme activity (IC_{50} value) of freshly prepared human and rodent AChE, and butyrylcholinesterase (BChE) was quantified at half-log concentrations ranging between 0.3 nM and 30 μM, as described previously (Yu et al., 1997). Acetyl-(*b*-methyl)thiocholine and *s*-butyrylthiocholine were used as substrates for AChE and BChE, respectively, and posiphen and phenserine assays were performed side by side using (−)-physostigmine as an external control because its activity is well known. Analysis of anticholinesterase activity was determined at 37°C in pH 8.0 phosphate buffer (0.1 M), the optimal working pH of both enzymes. Incubation was performed in the presence of 5,5′-dithiobis (2-nitrobenzoic acid) and production of a yellow thionitrobenzoate anion was measured by spectrophotometry at 412-nm wavelength. The mean enzyme activity determined at each drug concentration was subtracted from background (nonspecific binding) levels and expressed as a percentage of activity associated with the absence of compound. This was transformed into a logit format to allow calculation of an IC_{50} value, which was determined from a correlation between log drug concentration and logit activity. Only results obtained from correlation coefficients of $r^2 > 0.98$ were considered acceptable, and studies that did not obtain this threshold were repeated in full.

Statistical Analysis of the Data. Results from all experiments were analyzed with SAS 9.1 (SAS Institute, Cary, NC), and significance of difference from control was determined by Dunnett's multiple Student's *t* test against a common mean after first determining overall significance of $p \leq 0.01$ through analysis of variance. Drug response modeling was done excluding the control (0 mg/kg) samples. ED_{50} represented the dose in milligrams per kilogram required to induce a 50% of maximal drug-induced response in animal experiments and was calculated via the SAS NLIN procedure using the Hill equation model for the log of ED_{50}. EC_{50} values represented the concentration required to induce a 50% of maximal drug-induced response in cell culture experiments and were determined from a correlation between log concentration versus logit of activity: ln[activity/(100% − activity)].

Results

Posiphen and Phenserine Treatment of Human Neuroblastoma Cells. Administration of posiphen (10 and 50 μM) and phenserine (50 μM) to SK-N-SH neuroblastoma cells reduced their intracellular and secreted levels of APP at 16 h (for all doses, $p < 0.05$; Dunnett's Student's *t* test), as quantified by Western blot analysis (Fig. 2A). Significant change in cell viability was not observed. Secreted and intracellular levels of APP were decreased by approximately 40%, whereas levels of secreted Aβ, quantified by sandwich ELISA, were decreased by 21 and 32% by posiphen (10 and 50 μM) and by 38% by phenserine (50 μM) ($p < 0.05$) (Fig. 2B). APP mRNA levels were assessed by Northern analysis and were not significantly affected by either agent ($p > 0.05$).

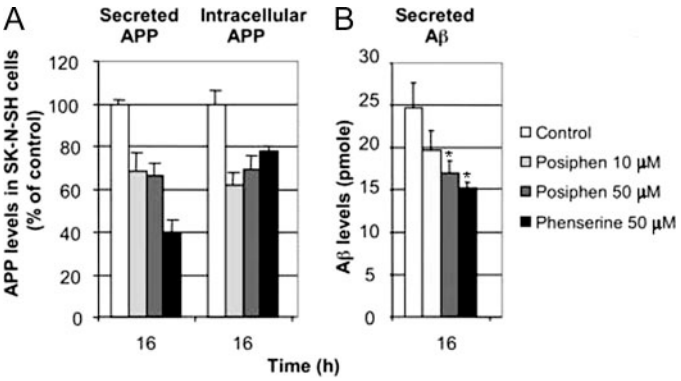

Fig. 2. Posiphen effects on APP and Aβ levels in human SK-N-SH cells. Concentration-dependent action of posiphen on secreted and intracellular APP levels (A) and secreted Aβ levels (B) in human neuroblastoma (SK-N-SH) cells in culture at 16 h of treatment (mean ± S.E.M.). A, all levels reduced compared with controls; B, 16-h levels at 50 μM reduced compared with controls ($p < 0.05$, Dunnett's). Levels of APP and Aβ were measured by Western blotting and sandwich ELISA, respectively, as described in the text.

To define concentration dependence, SH-SY-5Y neuroblastoma cells were treated for 16 h with posiphen and phenserine (0.2–20 μM) or vehicle, secreted, and intracellular levels of APP were quantified. Both agents induced a similar concentration-dependent decline in APP levels by approximately 50%, beyond which increases in dose resulted in no further APP reductions. The concentration required to induce a 50% decrease in the maximal drug-induced effect (EC_{50}) were similar for both agents and were 1.0 and 0.64 μM for posiphen and phenserine versus extracellular (secreted) APP and 1.5 and 1.14 μM for posiphen and phenserine versus intracellular (membrane-bound) APP (Fig. 3, A and B).

A decline in secreted and intracellular APP levels in the absence of a corresponding decrease in APP mRNA can be due to a change in posttranscriptional regulation through a reduction in translational efficiency. To evaluate translational effects, levels of newly synthesized APP were determined by a brief, 10-min incubation in the presence of ^{35}S-labeled amino acids. Thereafter, APP protein was immunoprecipitated, and the amount of radiolabel incorporated was normalized by TCA-precipitable counts. Both posiphen (10 μM) and phenserine (10 μM) lowered levels of newly synthesized APP by approximately 50% ($p < 0.05$, Dunnett) (Fig. 4, A and C). In contrast, APP mRNA levels were unaffected ($p > 0.05$, Dunnett) (Fig. 4D), as were total protein levels (assessed by TCA-precipitable counts; Fig. 4B).

Action of Posiphen and Phenserine on Cholinesterase Enzymes ex Vivo. The concentration of posiphen and phenserine required to inhibit 50% enzyme activity (IC_{50}) of freshly prepared human and rodent AChE and BChE is shown in Table 1. The agents possessed similar IC_{50} values in both species. However, although phenserine proved to be a potent and AChE selective inhibitor, posiphen lacked inhibitory action against both enzyme subtypes.

Gross Effects of Posiphen and Phenserine in Mice. Mice administered posiphen (7.5–75 mg/kg) and phenserine (2.5 and 7.5 mg/kg) once daily for 21 consecutive days showed weight gains similar to controls. The sole gross behavioral effect was a centrally mediated fine tremor in animals administered 7.5 mg/kg phenserine, which lasted for up to 3 h postadministration.

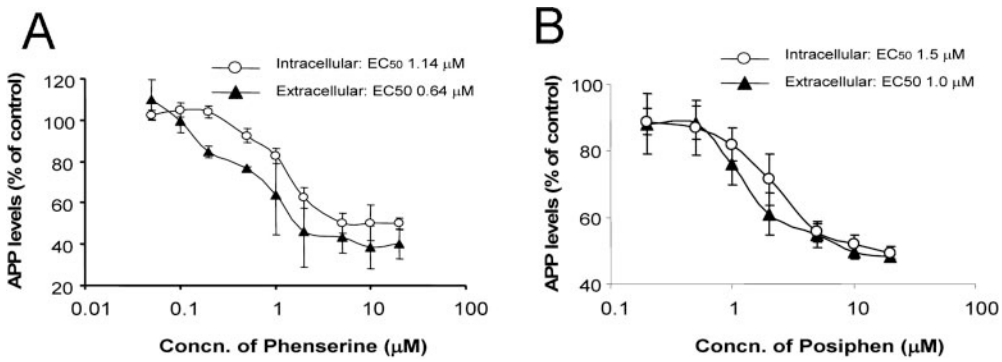

Fig. 3. Concentration-dependent action of posiphen on APP levels in human SH-SY-5Y cells. Concentration-dependent action of phenserine (0.2–20 μM) (A) and posiphen (0.2–20 μM) (B) on secreted and intracellular APP levels in human neuroblastoma (SH-SY-5Y) cells in culture (mean ± S.E.M.) as assessed at 16 h. Intra- and extracellular levels of APP were maximally reduced by approximately 50%, and EC_{50} values relating to this reduction were 1.0 and 0.64 μM for posiphen and phenserine versus secreted APP, and 1.5 and 1.14 μM for posiphen and phenserine versus intracellular APP. Data for this represent levels of APP quantified from Western blots.

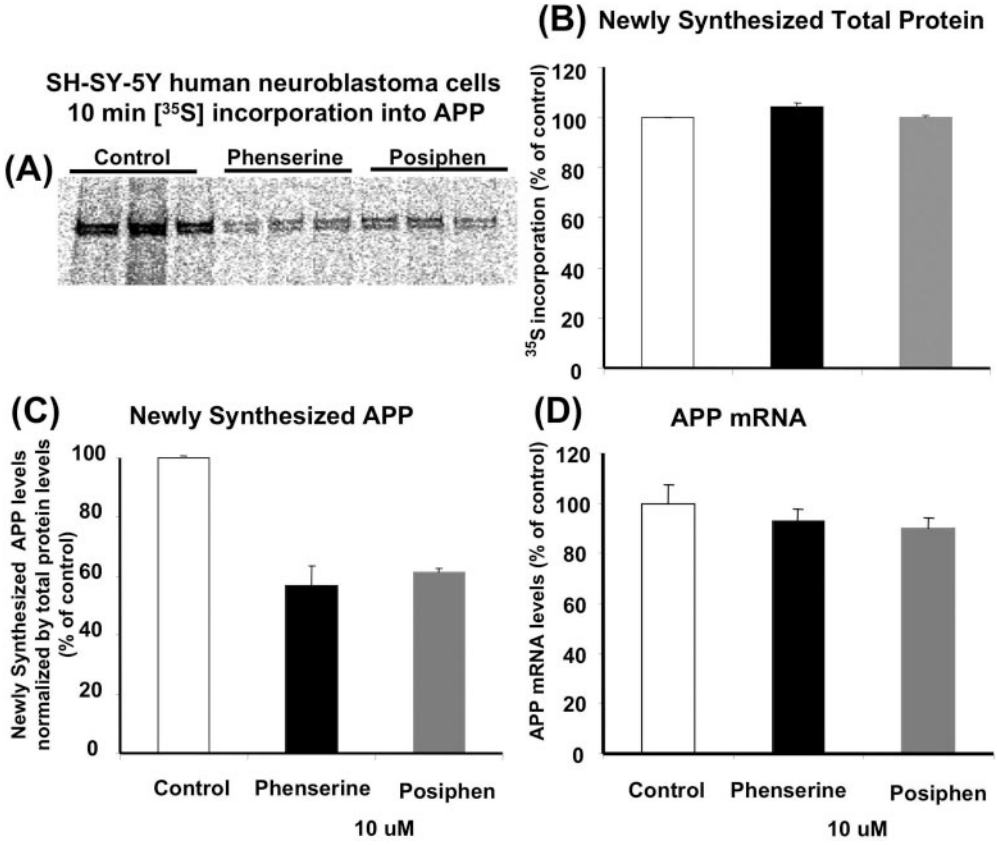

Fig. 4. Translational regulation by posiphen and phenserine (rate of APP synthesis) in SH-SY-5Y cells. A, translation was assessed by addition of radiolabeled amino acids for 10 min followed by immunoprecipitation of the newly synthesized APP protein. B, newly synthesized total protein was assessed by ^{35}S-incorporated protein levels (TCA-precipitable counts) normalized by nonprecipitable counts and was unaffected by posiphen and phenserine (10 μM). C, newly synthesized APP levels were then normalized by ^{35}S-incorporated proteins. Posiphen and phenserine (10 μM) both and to a similar extent significantly decreased newly synthesized APP levels (50% reduction, $p < 0.05$, Dunnett). D, APP mRNA levels were assessed by reverse transcriptase-PCR. Treatment with posiphen and phenserine did not affect APP mRNA levels ($p > 0.05$, Dunnett).

TABLE 1
Effects of phenserine and posiphen on human and rodent AChE and BChE inhibition

Treatment	AChE Inhibition (10 μM)	AChE (IC_{50})*	BChE Inhibition (10 μM)	BChE (IC_{50})
Phenserine (human)	100%	22 ± 4 nM	100%	1.8 ± 0.4 μM
Phenserine (rodent)		26 ± 5 nM		2.2 ± 0.2 μM
Posiphen (human)	<15%	16 ± 2 μM	<8%	28 ± 1 μM
Posiphen (rodent)		20 ± 2 μM		30 ± 3 μM

* IC_{50}, concentration required to inhibit 50% enzyme activity.

Drug Action on Cerebral Cortex Hemisphere Mass and Total Protein per Brain Tissue Mass. Phenserine (2.5 and 7.5 mg/kg) had no significant effect on either brain hemisphere mass (Fig. 5A) or total protein content per brain tissue mass (Fig. 5B). In contrast, a dose-dependent, significant relationship between posiphen dose and brain hemisphere mass ($r = -0.41978$, $p < 0.01$) was found in posiphen treated animals (7.5–75 mg/kg). However, the individual treatment difference from control was only significant at 75 mg/kg (control, 173 ± 2.59 mg versus posiphen, 190 ± 2.84

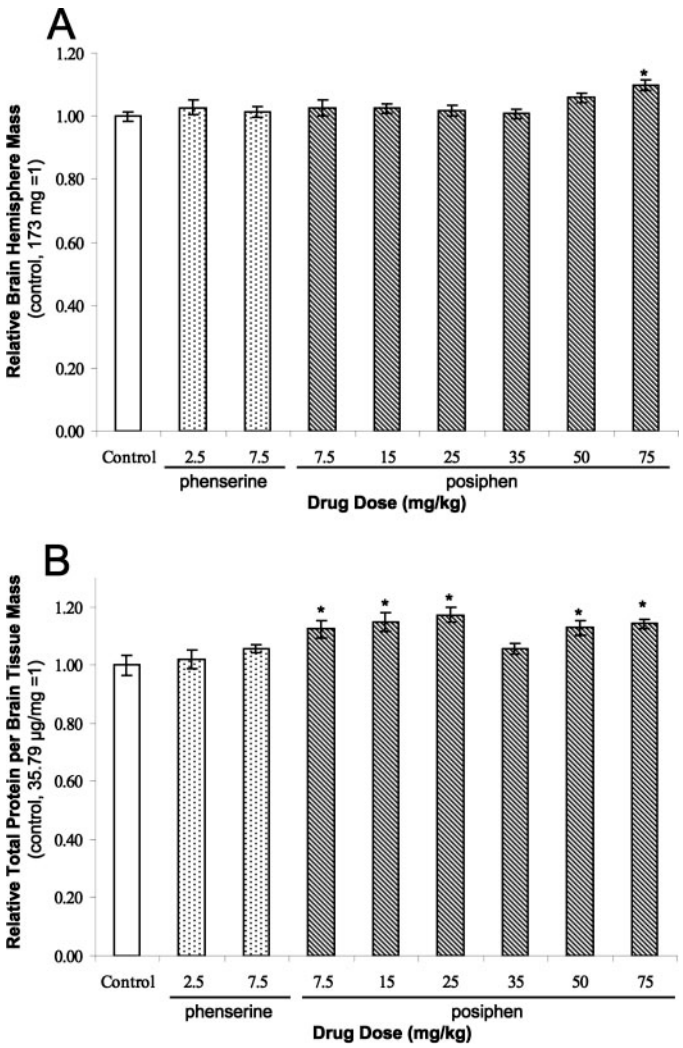

Fig. 5. Brain hemisphere mass and total protein per brain hemisphere. White bars, control (saline i.p.) mice. Stipple, phenserine-treated mice. Diagonal hatch, posiphen-treated mice. Error bars, S.E.M. A, right hemispheres of treated mice were harvested and weighed. Results were adjusted to the mean mass of control mice brain hemispheres. *, Samples significantly different from control at $p < 0.05$. B, harvested cerebral hemispheres of treated mice were homogenized in buffer as described in the text and protein content measured. Results were adjusted to the mean protein content of control mice brain hemispheres. *, samples significantly different from control at $p < 0.05$.

mg, $p < 0.05$, Dunnett). In contrast, all posiphen treatments, except 35 mg/kg, had a significantly greater protein content than did control samples (control, 35.79 ± 1.18 µg protein/mg brain tissue versus posiphen, 40.22 ± 1.09 to 42.00 ± 0.84 µg/mg, $p < 0.05$). This trend was not dose-dependent. Because posiphen administration was associated with a greater significant effect on total protein than on tissue mass, further measurements (e.g., quantification of APP and Aβ) were adjusted to brain tissue mass, which is likely to underestimate the changes observed.

Drug Action on Levels of Total APP Mouse Cerebral Hemisphere. Western blots of extracts from mouse brain hemispheres were probed for total APP (Fig. 6A) and β-actin (Fig. 6B), and levels were quantified (Fig. 7). Phenserine treatment (7.5 mg/kg) lowered levels of β-actin-adjusted APP, normalized to tissue mass, without attaining significance ($p > 0.05$, Dunnett). A dose-dependent decline in levels of adjusted APP was achieved by posiphen (Pearson's $r = -0.38517$, $p < 0.01$, $n = 57$) (Fig. 7A). Adjusted APP was decreased by 40%, versus control, by 35 mg/kg posiphen and declined by a maximal 50% at higher doses (posiphen ≥ 35 mg/kg, $p < 0.05$). Nonlinear modeling to the standard sigmoid curve generated an ED_{50} of 19 mg/kg (Fig. 7B), $p = 0.01$.

Drug Action $A\beta_{40}$ and $A\beta_{42}$ Levels in Mouse Cerebral Cortex. A slight, but nonsignificant, decline in $A\beta_{40}$ levels was achieved by both phenserine doses (Fig. 8). In contrast, 7.5 mg/kg posiphen significantly lowered $A\beta_{40}$ (control, 14.53 ± 0.42 pg $A\beta_{40}$/mg brain tissue versus posiphen, 12.44 ± 0.47 pg/mg, $p < 0.05$, Dunnett), and higher doses induced further dose-dependent declines ($r = -0.64243$, $p < 0.001$, $n = 56$) to a maximal 40% drop. In comparison, all doses of phenserine and posiphen significantly reduced brain $A\beta_{42}$ levels ($p < 0.05$) by up to 33% for phenserine (7.5 mg/kg) and 58% for posiphen (25 mg/kg), albeit with no apparent dose dependence ($r = -0.12592$, $p < 0.399$, $n = 47$) (Fig. 9). The correlation coefficients for posiphen treatment effects on $A\beta_{40}$ levels versus $A\beta_{42}$ levels are significantly different from each other by Fisher's Z test ($p < 0.05$).

Drug Actions on Brain α-, β-, and γ-Secretase Activity. To determine the relative α-, β-, and γ-secretase activities, kits from R&D Systems were used without additional validation, recognizing that the assays may detect other proteases with similar specificities. β-Secretase activity (Fig. 10) was significantly reduced in brain extracts from posiphen-

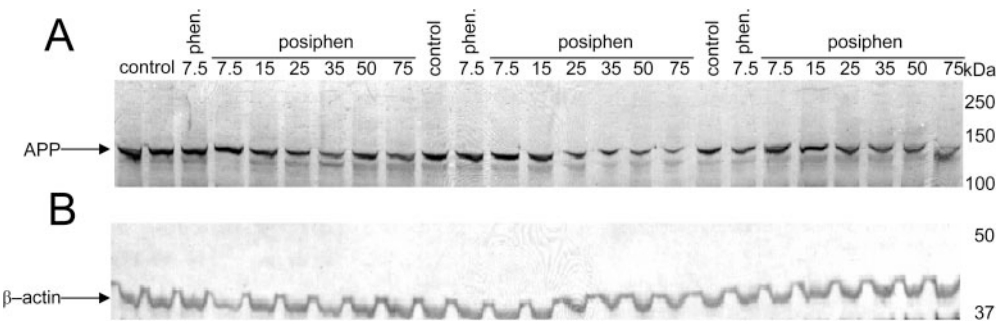

Fig. 6. Western blot of mouse brain extracts for APP and β-actin. Mouse brain extracts were run on SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with antibodies to APP or β-actin as described in the text. A, Western blot for APP. A monoclonal antibody against APP (22C11) was used to probe membrane. The rest of the procedures for Western blotting is as described in the text. APP band is indicated (left of blot), as are nearest molecular mass marker positions (right of blot). B, Western blot for β-actin. A monoclonal antibody against the β-actin protein was used to probe the membrane. The β-actin band is indicated (left of blot), as are nearest molecular mass marker positions (right of blot).

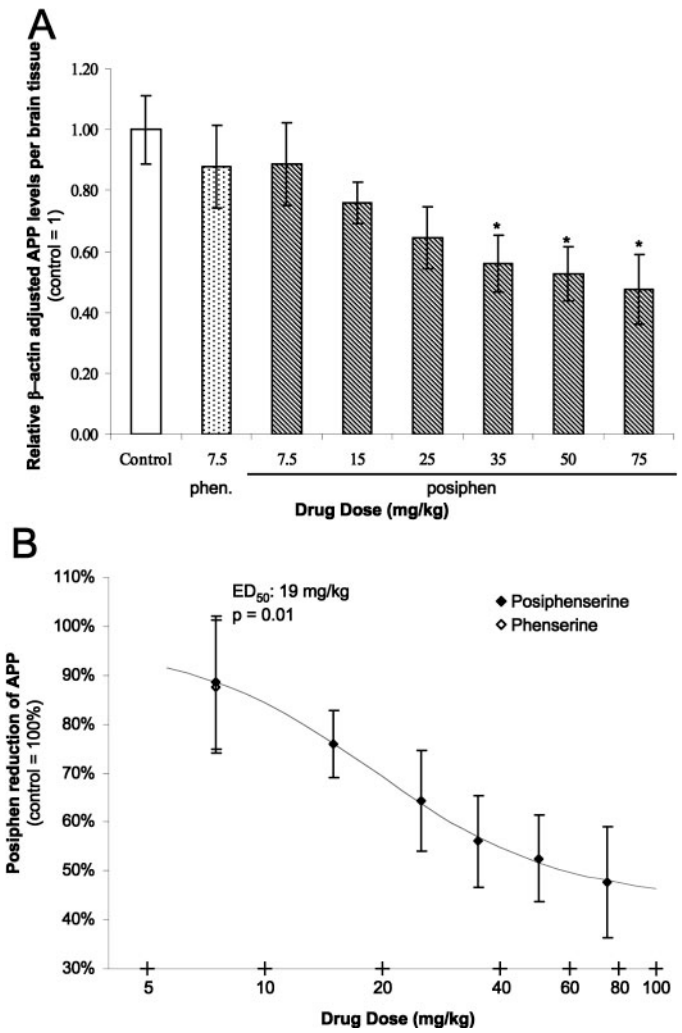

Fig. 7. Phenserine and posiphen treatment effects on APP levels. A, white bar, control (saline i.p.) mice. Stipple, phenserine-treated mice. Diagonal hatch, posiphen-treated mice. Error bars, S.E.M. APP was measured by Western blot and adjusted for actin and tissue mass as indicated in the text. *, samples significantly different from control at $p < 0.05$. B, dose-response model of posiphen activity on adjusted APP levels in mouse cerebral cortex hemispheres. Nonlinear regression was significant at $p = 0.01$. Phenserine dose result included for reference.

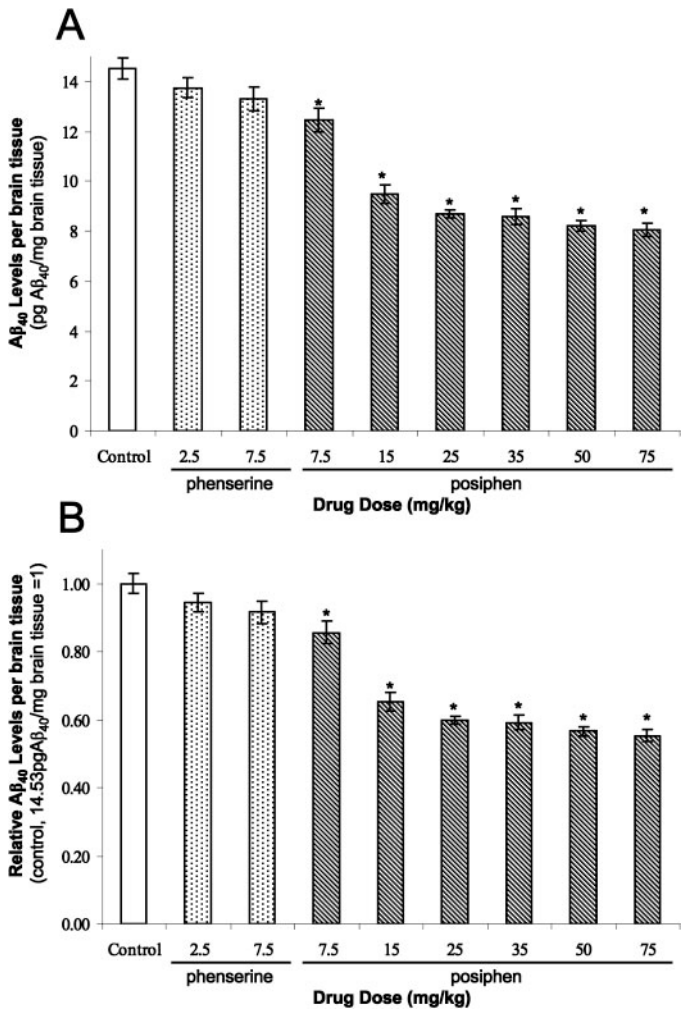

Fig. 8. Phenserine and posiphen dose effects on $A\beta_{40}$ levels in mouse cerebral cortex. White bars, control (saline i.p.) mice. Stipple, phenserine-treated mice. Diagonal hatch, posiphen-treated mice. Error bars, S.E.M. A, $A\beta_{40}$ levels in mouse cerebral cortex. Extracts were used for $A\beta_{40}$ ELISA as described in the text. Results were adjusted to $A\beta_{40}$ per brain tissue. *, samples significantly different from control at $p < 0.05$. B, relative $A\beta_{40}$ levels in mouse cerebral cortex (control = 1). *, samples significantly different from control at $p < 0.05$.

treated animals administered 35 and 50 mg/kg [control, 199.12 ± 17.85 fluorescence units (FU)/mg brain tissue versus 159.64 ± 6.31 FU/mg and 143.74 ± 7.97 FU/mg, $p < 0.05$, Dunnett]. Furthermore, the trend over all doses was dose-dependent by both tissue mass-adjusted ($r = -0.62417$, $p < 0.01$, $n = 28$) and raw fluorescence signal ($r = -0.56636$, $p < 0.01$, $n = 28$) measurements. In contrast, there was no trend associated with either α- or γ-secretase activity and phenserine or posiphen treatment (Fig. 11, A and B). The β-secretase assay signal exceeded that for the highest standard amount used (2 μg of β-secretase protein), indicating that the effect differences between doses may actually be greater than measured.

To define secretase assay specificity, heating of posiphen and control samples eliminated β-secretase activity (Fig. 12A). In addition, substrate specificity for the β-secretase assay was assessed in brain extracts (7.5 mg/kg posiphen) by performing the assay with the substrates for α-, β-, and γ-secretases (Fig. 12B). The β-secretase assay was active solely with its own substrate, providing no measurable activity with the α- and γ-secretase substrates.

Discussion

Prime hallmarks of AD are synaptic loss and abnormal protein deposition, particularly of toxic Aβ, derived from APP by the action of β- and γ-secretase enzymes (Selkoe, 2005). Hence, initial treatment strategies have primarily focused on improving cognitive processes through AChE inhibition and reducing brain levels of Aβ. Although presently approved anticholinesterases and memantine are primarily useful for symptomatic relief (Lahiri et al., 2003b), they seem to have a limited impact on disease progression and Aβ deposition. This has provided impetus to design agents that possess dual actions upon targets associated with cognition and mechanisms driving neuronal dysfunction and death in AD. Because both Aβ levels and cholinesterase activity are affected in the AD brain (Giacobini, 2003; Lahiri et al., 2003b; Lleo et al., 2006), a focus of our research has been to elucidate

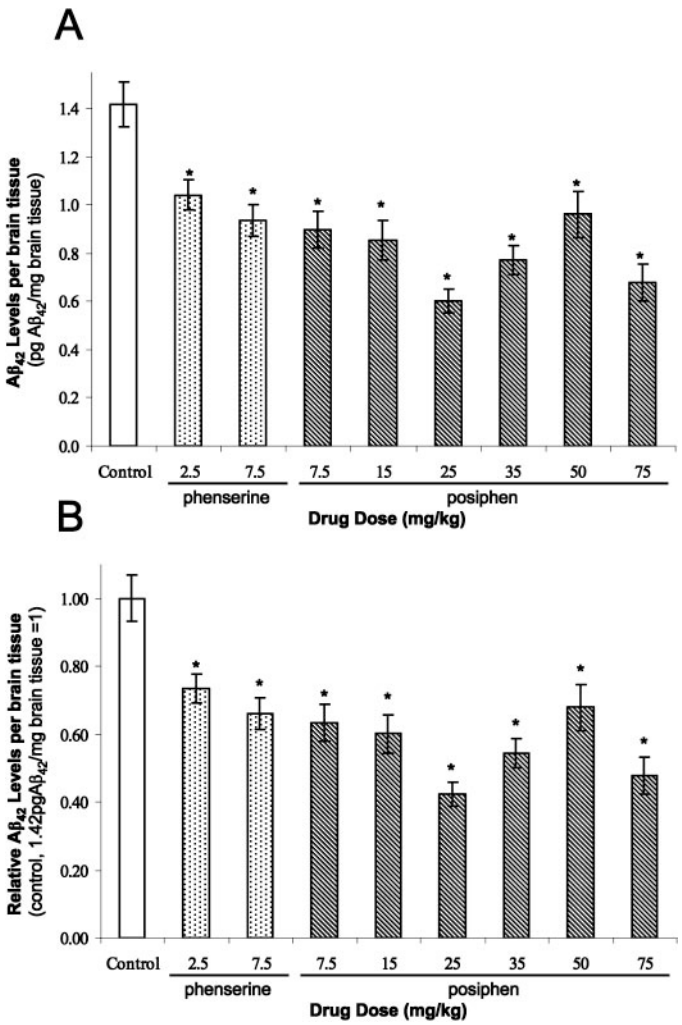

Fig. 9. Phenserine and posiphen dose effects on $A\beta_{42}$ levels in mouse cerebral cortex. White bars, control (saline i.p.) mice. Stipple, phenserine-treated mice. Diagonal hatch, posiphen-treated mice. Error bars, S.E.M. A, $A\beta_{42}$ levels in mouse cerebral cortex. Extracts were used for $A\beta_{42}$ ELISA as described in the text. Results were adjusted to $A\beta_{42}$ per brain tissue. *, samples significantly different from control at $p < 0.05$. B, relative $A\beta_{42}$ levels in mouse cerebral cortex (control = 1). *, samples significantly different from control at $p < 0.05$.

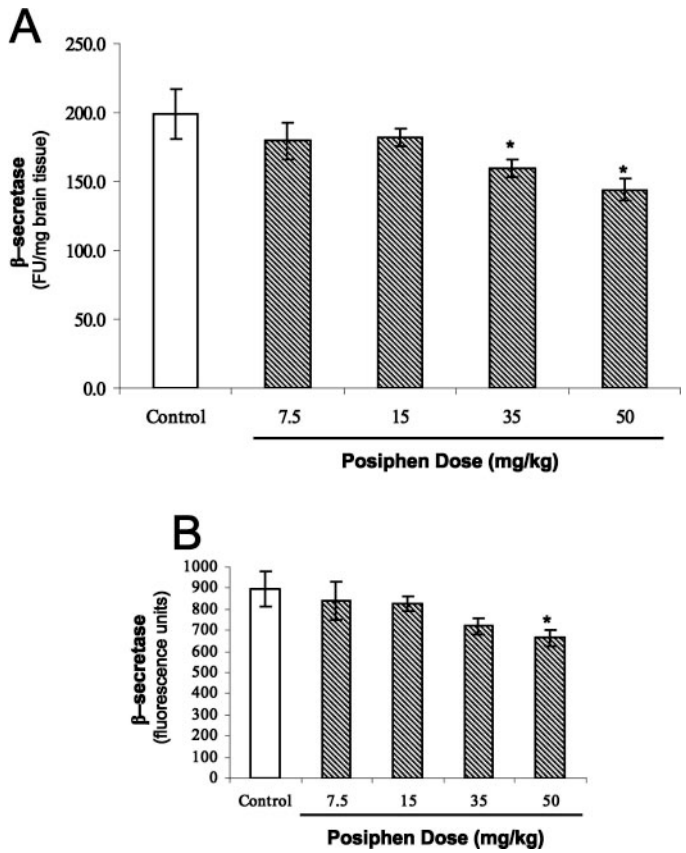

Fig. 10. Posiphen dose effects on β-secretase activity in mouse cerebral cortex. Mouse cerebral cortex hemisphere extracts were used for assay of β-secretase as described in the text. White bars, control (saline i.p.) mice. Diagonal hatch, posiphen-treated mice. Error bars, S.E.M. A, β-secretase activity in mouse cerebral cortex, on a fluorescence unit per milligram of brain tissue basis. *, samples significantly different from control at $p < 0.05$. B, β-secretase assay raw FU.

whether or not common molecular mechanisms link the two targets (Lahiri et al., 1994, 1998, 2003a; Shaw et al., 2001). This work culminated in the development of carbamates on the backbone of hexahydropyrrolo[2,3*b*]indole (Yu et al., 1997) and the development of one of these, phenserine, to AD clinical trials (Greig et al., 2005; Lahiri et al., 2006).

We have reported previously that phenserine is a potent acetyl subtype-selective anticholinesterase that lowers APP levels in neuronal cell cultures at the post-transcriptional level (Shaw et al., 2001), activities that are supported by the present study. This activity is distinct from that of muscarinic agonists, which stimulate α-secretase cleavage of APP and selectively lower the yield of Aβ. Prospective regulation of APP at the level of translation was initially inferred from studies involving reversible ischemia in rabbit spinal cord, where APP levels were elevated as early as 15 and 60 min without changes in APP mRNA expression (Wallace et al., 1995); and cholinergic forebrain (nucleus basalis) lesions in rats that resulted in a rapid, 1-h, and sustained elevation of APP and Aβ in cortex and cerebrospinal fluid (Wallace et al., 1991; Haroutunian et al., 1997). This latter model mimics the forebrain cholinergic loss found in AD. Because an increase in protein stabilization would result in a decrease in proteolytic peptides, the rise in APP and Aβ levels in the rodent cholinergic forebrain lesion model probably results from actions occurring before protein synthesis. Furthermore, the rapidity of the elevation eliminates transcriptional induction that has been estimated to require 4 h for the APP gene. Hence, the increase in APP protein is probably due to increased translational efficiency of the mRNA. Further studies demonstrated that this rise in APP could be eliminated by treatment of rats with phenserine (Haroutunian et al., 1997), suggesting that phenserine may act at the translational level to reduce APP synthesis. In addition, we have recently identified several novel small inhibitors of APP protein synthesis, which can be used to lower amyloid-β peptide levels (Utsuki et al., 2006).

The present studies support this concept demonstrating consistent posiphen and phenserine-induced concentration-dependent reductions of i) APP protein levels in neuronal cultures, as assessed by measuring steady-state levels after 16 h, and ii) the rate of APP synthesis, as determined by quantifying ^{35}S incorporation into APP after immunoprecipitation of cell extracts obtained after a 10-min pulse label. These studies detected an up to 50% reduction in the synthe-

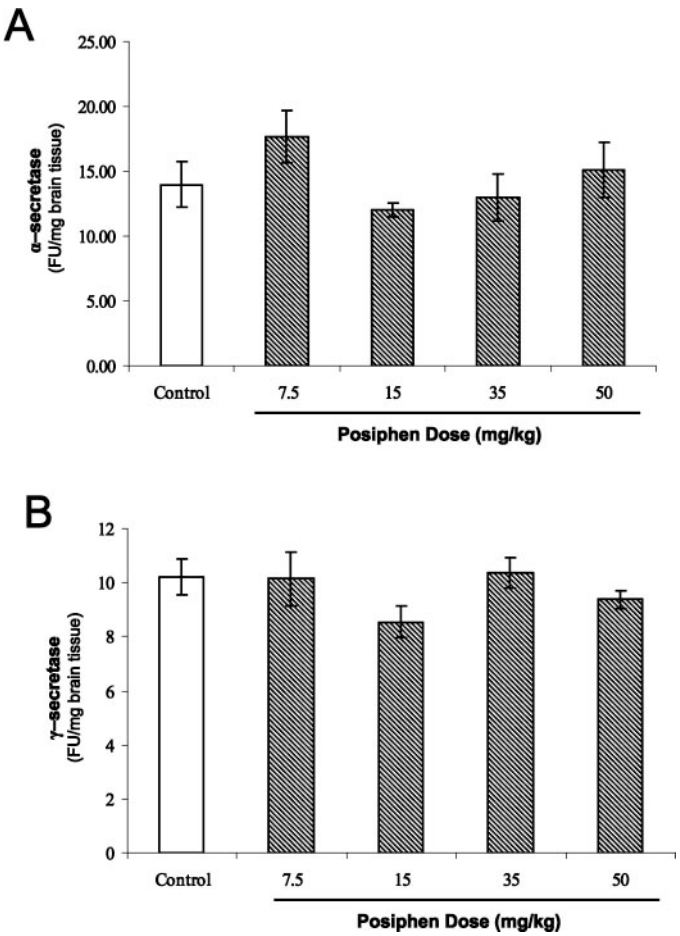

Fig. 11. Posiphen dose effects on α-secretase and γ-secretase activity in mouse cerebral cortex. A, mouse cerebral cortex hemisphere extracts were used for assay of α-secretase as described in the text: α-secretase activity in mouse cerebral cortex, on a fluorescence unit per milligram of brain tissue basis. B, mouse cerebral cortex hemisphere extracts were used for assay of γ-secretase as described in the text: γ-secretase activity in mouse cerebral cortex, on a fluorescence unit per milligram of brain tissue basis. White bars, control (saline i.p.) mice. Diagonal hatch, posiphen-treated mice. Error bars, S.E.M. No samples significantly differed from control.

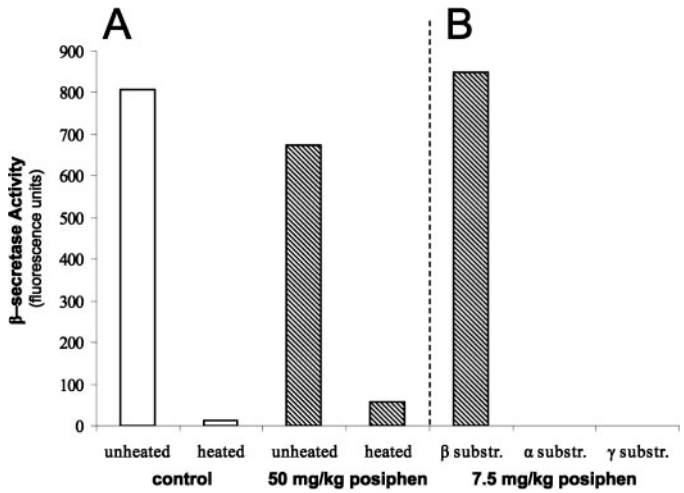

Fig. 12. Specificity of β-secretase assay. A, control and posiphen-treated cortical hemisphere extracts and heated extracts were assayed with the β-secretase assay as described in the text. White bar, activity for control samples. Diagonal hatch, activity for posiphen-dose samples. B, cortical hemisphere extracts from mice treated with 7.5 mg/kg posiphen were assayed with the β-secretase assay, using the substrates for α-, β-, and γ-secretases each as substrates, as described in the text.

sis of APP without detectable alteration of either APP mRNA or total protein synthesis. Interestingly, the EC_{50} required to induce a decline in secreted and intracellular APP, ranging between 0.64 and 1.5 μM for both agents, is some 30-fold higher than the IC_{50} defining the AChE inhibition of phenserine, but almost 25-fold lower than its cholinergically deficient enantiomer, posiphen, allowing the use of larger doses of the latter. Although actions on APP and Aβ translated from culture to in vivo for both compounds were similar at the same dose, phenserine-mediated reductions were constrained by its cholinergic activity, a centrally mediated tremor that was dose-limiting at 7.5 mg/kg. This action is consistent with the agent's high brain/plasma ratio and elevation of brain acetylcholine levels (Greig et al., 2005). In comparison, a log greater amount of posiphen was administered without visible consequences on gross behavior, weight, or general appearance.

Posiphen- and phenserine-induced declines in brain APP were highly dose-dependent. However, similar to cell culture studies, a maximal decline of 50 to 60% was achievable, beyond which further increases in dose were without effect. These reductions in brain APP, likewise, translated into lowered levels of brain Aβ that also showed dose dependence up to a decline of 50 to 60% in this particular study. Drug-induced dose-dependent declines in brain APP and $A\beta_{40}$ were similar, whereas those for $A\beta_{42}$ were more robust. As an example, declines in APP, $A\beta_{40}$, and $A\beta_{42}$ were for 7.5 mg/kg phenserine, 13, 7.5, and 33%; and for 7.5 mg/kg posiphen, 11, 14, and 37%, suggesting a slightly greater effect on the latter form at low doses, in accord with the significantly different Fisher's Z test analysis of dose dependence. The level of $A\beta_{42}$, the less water-soluble and more toxic of the Aβ forms, is particularly elevated in the AD brain (Sambamurti et al., 2002). However, whether or not this preferential reduction translates to humans remains to be determined, and current studies are quantifying phenserine's time- and dose-dependent actions on plasma APP, $A\beta_{40}$, and $A\beta_{42}$ levels from clinical samples.

We have previously reported that phenserine lowers the translational efficiency of APP in U373 astrocytoma cells via a 90-nucleotide sequence spanning +55 to +144 (+1 being the transcription start site) from the 5′-cap site, within the 5′-UTR of APP mRNA (Shaw et al., 2001). This same region contains an IL-1-responsive element, a transforming growth factor-β-responsive element, and an iron-responsive element, all of which, in reaction to appropriate ligands, can up-regulate APP levels (Rogers et al., 1999; Lahiri et al., 2003a). Further elements regulating APP translational efficiency have also been identified in the 3′-UTR and 5′-UTR of APP mRNA (Mbella et al., 2000). Protein synthesis entails a complex process involving ribosome assembly, initiation and elongation. Although the efficiency of mRNA translation can be controlled at any of these points, initiation, which includes the level and activity of initiation factors, primarily represents the major regulatory step (Holcik and Sonenberg, 2005; Pickering and Willis, 2005).

Protein translation has a particularly critical role in dendritic function and synaptic plasticity within the nervous system (Sutton and Schuman, 2005), where changes in syn-

aptic efficacy, requiring immediate changes in gene products, occur within localized specific regions that are generally distant from their cell body. Indeed, several biochemical signaling cascades have been demonstrated to couple neurotransmitter and neurotrophin receptors to translational regulatory factors to match both specific and general protein synthesis to local synaptic requirements (Klann and Dever, 2004). It remains to be elucidated whether or not phenserine and posiphen, in addition to numerous proinflammatory cytokines that are elevated in AD brain (Klegeris and McGeer, 2005), share these.

Our results suggest that β-secretase activity can also be regulated by posiphen because its level of activity was reduced in the brains of treated mice with reduced levels of $A\beta_{40}$ and $A\beta_{42}$. No actions were evident on either α- or γ-secretase activities. In an additional preliminary study, after treatment of rat fetal primary brain cortical cell cultures with posiphen, we have further observed secreted APP but not total APP to be reduced (J. Bailey and D. K. Lahiri, unpublished data). Like APP, β-secretase expression and activity can be up-regulated by inflammatory stimuli (Sastre et al., 2006) and oxidative stress in culture as well as in brain of sporadic AD patients (Holsinger et al., 2002). BACE1 expression can be regulated at the transcriptional and translational level (Ge et al., 2004; Rogers et al., 2004), with nonsteroidal anti-inflammatory drugs lowering levels via the former (Sastre et al., 2006). The mechanisms underpinning posiphen's action remain a focus of interest. Recent studies indicate that a small rise in BACE1 can induce a dramatic elevation in Aβ production (Li et al., 2006), and whether a small reduction will initiate the reverse remains to be determined. We plan to examine this valuable potential property of posiphen as a lead compound for β-secretase inhibition in future studies.

The present results should be interpreted with some caveats. Herein, we observed a 10% increase in brain mass with posiphen treatment that attained significance. However, it should be noted that this increase was observed only in the highest dose of the drug, and its biological significance needs to be determined. In these experiments, the maximal reduction in Aβ levels achieved was approximately 50%, and it is not known why the reduction leveled off around 50% from the control. However, in another set of animal experiments at a different institution, the reduction in Aβ level was found to be smaller (~10%) but significant with posiphen versus control. Such variation could be due to the nature of animals, solubility/stability of the drug, assay conditions, or other unknown factors. Regarding enzymatic processing of APP, the reduction in β-secretase activity could be either exclusively due to direct drug action on this enzyme or reduction of other proteases that may also operate upon the specific substrate used in our assay. For this, a careful examination of the effect of posiphen on levels of different carboxyl truncated fragments of APP, such as CTFβ and CTFγ species, needs to be performed. From our animal data, the drug should be tested in an animal model of AD for verification of this difference as well as tests of learning and memory.

Taken together, our results suggest that posiphen is a promising experimental AD drug that, together with phenserine, has provided a useful pharmacophore in the design of potentially novel Aβ-lowering agents. Both agents seem to work on several levels, possessing a regulatory action on APP synthesis at a translational level that lowers its proteolytic products, especially AB_{42}. However, posiphen is unencumbered by dose-limiting cholinergic overdrive, which is common to AChE inhibitors. It hence represents a compound that warrants assessment in AD as a single agent and potentially in combination with an anticholinesterase, such as phenserine. Moreover, as a unique agent that lowers β-secretase as well as APP, posiphen sets a unique paradigm for drug development.

Acknowledgments

Animal studies were conducted under an approved Institutional Animal Care and Use Committee protocol.

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