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The Experimental Alzheimer's disease drug Posiphen lowers amyloid-β peptide levels in cell culture and mice

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Running Title: Posiphen lowers amyloid-β levels *in vivo*

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Nonstandard abbreviations. 5'-UTR, 5'-mRNA untranslated region; Aβ, amyloid-β peptide; AD, Alzheimer's disease; AChE, acetylcholinesterase; AChEI, AChE inhibitor; APP, Aβ precursor protein; BChE, butyrylcholinesterase; ChEI cholinesterase inhibitor; FCS, fetal calf serum; LDH, lactate dehydrogenase; NB, SK-N-SH human neuroblastoma; posiphen, (+)-phenserine; τ, microtubule-associated protein tau; ADDLs, Aβ derived diffusible ligands.

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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 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, (+)-phenserine (posiphen), was assessed. In cultured human neuroblastoma cells, posiphen, like phenserine, dose- and time-dependently lowered APP and Aβ levels by reducing APP synthesis rate. This action translated to an *in vivo* system. Posiphen administration to mice (7.5 to 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 (≥15 mg/kg) compared to 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.

Introduction

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 39-43 amino acid peptide (Mw~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; 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 (ADDLs) 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 (LaFerla and Oddo, 2005; Walsh et al., 2002). 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 reduction as a strategy for the treatment of AD (LaFerla and Oddo, 2005). Nevertheless, since 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 (CTFβ) 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). Whereas 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 tau 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 US (Giacobini, 2003), to which the recently approved NMDA antagonist, memantine, has been added (Lleo et al., 2006). Whereas AChE inhibitors are widely considered to be symptomatic drugs that elevate brain acetylcholine (ACh) 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., 1998; Lahiri et al., 1994; 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; Greig et al., 2005). The regulation of APP expression represents an important untapped approach to AD treatment, as 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) (Figure 1), a physostigmine analogue 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 (IRE), interleukin-1 (IL-1) element (Rogers et al., 1999), and a transforming growth factor-β1 (TGFβ) response element (Lahiri et al., 2003a) that are all elevated in AD brains (Venti et al., 2004). In addition, the IRE, IL-1 element, and a "CAGA" box participate in a structure similar to the "bulge-loop" structure of HIV-1 TAR RNA (Maloney et al., 2004).

A potential concern for dual action drugs that inhibit AChE is that this latter action is generally dose-limiting. Therefore, we investigated whether (+)-phenserine (posiphen) (Figure 1), a chiral isomer largely devoid of anticholinesterase activity (Greig et al., 2005; Yu et al., 1997), 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.

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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 PBS for in vivo work or in the respective medium for cell culture studies.

Most chemicals were purchased from Sigma (St Louis, MO, USA) 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 either Roche (Indianapolis, IN, USA), Chemicon (Temecula, CA, USA) or Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Culture. Human neuroblastoma cell lines SK-N-SH and SH-SY-5Y were obtained from the American Type Culture Collection, culture medium was from Mediatech, Inc. (Herndon, VA) and the fetal calf serum (FCS) 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 x 10^6 cells, respectively. Cells were allowed to grow in complete media (10 % FCS, 2 mM glutamine in DMEM) for 2 days to reach 70% confluence. Thereafter, spent media was removed and replaced with fresh media (2 ml 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. Fifteen μg of protein from each sample was mixed with Laemmli buffer, boiled for 5 min at 100 °C, and loaded onto a 10% SDS-PAGE gel (Novex, San Diego, CA). The proteins separated at 150V for 90 min, transferred to nitrocellulose membrane

and probed with 22C11 (2 µg/ml) antibody to APP. The monoclonal 22C11 antibody recognizes 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 chemiluminesence. Cell viability was assessed by measurement of lactate dehydrogenase (LDH) levels (Lahiri et al., 1998). Levels of Aβ from conditioned medium were quantified by ELISA. Rabbit polyclonal antibody #3160 ($A\beta_{40}$) was used as a capture antibody for all species of Aβ ($A\beta_{40}$ and $A\beta_{42}$) while monoclonal antibody 4G8 ($A\beta_{17\text{-}25}$) 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 hr) 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 of glutamine for 1 hr. 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, 10% glycerol) containing protease inhibitors (PMSF, 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 for 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 phosphor screen (Perkin/Elmer, Wellesley, MA) overnight and the APP signal was quantified on phosphorimager. The levels of newly synthesized APP were normalized

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by [^{35}S] incorporated protein levels, which are TCA precipitable counts normalized by TCA non-precipitable 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 sub-saturating level. Using the optimal concentration of antibody, APP signals were detected between 100 and 120 kDa molecular weight and no other signal as 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 GAPDH 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 RT-PCR was carried out with the PE Applied Biosystems Prism 7700 Sequence Detection System using the following parameters: 1 cycle of 2 min at 50°C and 10 min at 95°C, and 40 cycles of 15 seconds at 95°C and 60 seconds 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 cycles (CT) value) were calibrated to relative RNA (APP or GAPDH) 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 phosphorimage analysis on the Cyclone phosphorimager (Perkin/Elmer).

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Drug treatment in the animal. Adult male mice (C57Blk), 28 to 32 g weight, were obtained from Harlan Labs (Indianapolis, IN, USA) and had free access to food and water. In accord with an approved the Institutional Animal Care and Use Committee (IACUC) 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 90-120 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 (MAD) showed that two samples (one control and one posiphen 7.5 mg/kg) were beyond 4 MAD 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 manufacturer's instructions for enzymatic assay kits (R&D Systems, Inc, Minneapolis, MN). Briefly, the brain cortices were homogenized in 800 µl low salt containing 1× extraction buffer (R&D kit) and incubated on ice for 10 minutes. The homogenates were then centrifuged at 13,000×g for 15 minute at 4°C. The protein concentration in each sample was estimated according to biuret-derived assay (Pierce Biotechnology, Rockford, IL). Levels of APP, $A\beta_{40}$, and $A\beta_{42}$ 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 8 treatments: control; phenserine 7.5; posiphen 7.5; 15; 25; 35; 50 and 75 mg/kg dose. To

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minimize intragel variation, each gel contained 3 sets of 8 treatment groups (set n = 3; total samples = 24). Additional gels/blots were performed to a total of 10 independent samples (except for control, and posiphen 7.5 and 75 mg/kg, 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 2 ways: blotwise (n = 3) to test for gel to gel variation and combined blots (n = 9 or10). Blotwise analysis with n = 3 showed the same trend as the final analysis with n = 9 or10 did. Results of the combined analysis were presented herein.

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

Assay of levels of $A\beta_{42}$ peptides in mouse cerebral cortex extracts. Brain extracts used in this assay were the ones that were previously tested in APP and $A\beta_{40}$ assays. A sensitive sandwich ELISA was performed to quantitatively assay levels of $A\beta_{42}$ in different brain extracts samples under the same condition as described (Basha et al., 2005; Lahiri et al., 2004). The procedure is otherwise as described for measuring $A\beta_{40}$ peptide levels.

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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 hours 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. Following incubation, the plates were analyzed for fluorescence at 355nm/500nm (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, as 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 (non specific binding) levels, and expressed as a percent 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 *t* test against a common mean after first determining overall significance of $p \leq 0.01$ through ANOVA. Drug response modeling was done excluding the control (0mg/kg) samples. ED_{50} represents the dose in mg/kg 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 *t* test), as quantified by Western blot analysis (Figure 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) ($p < 0.05$) (Figure 2B). APP mRNA levels were assessed by Northern analysis and were not significantly affected by either agent ($p > 0.05$).

To define concentration-dependence, SH-SY-5Y neuroblastoma cells were treated for 16 hr with posiphen and phenserine (0.2 to 20 μM) or vehicle, and 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 (Figure 3A 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) (Figure 4A and B). In contrast, APP mRNA levels were unaffected ($p > 0.05$, Dunnett) (Figure 4D), as were total protein levels (assessed by TCA-precipitable counts).

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 butyrylcholinesterase (BChE) is shown in Table 1. The agents possessed similar IC_{50} values in both species. However, whereas 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 to 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 phenserine 7.5 mg/kg, which lasted for up to 3 hr post administration.

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 (Figure 5A) or total protein content per brain tissue mass (Figure 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 to 75 mg/kg). However, the individual treatment difference from control was only significant at 75 mg/kg (control: 173 ± 2.59 mg vs. posiphen: 190 ± 2.84 mg, $p < 0.05$, Dunnett). In contrast, all posiphen treatments, except 35

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mg/kg, had a significantly greater protein content than did control samples (control: 35.79 ± 1.18 µg protein/mg brain tissue vs. posiphen: 40.22 ± 1.09 to 42.00 ± 0.84 µg/mg, $p < 0.05$). This trend was not dose-dependent. As 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 (Figure 6A) and β-actin (Figure 6B), and levels were quantified (Figure 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%, vs. control, by posiphen 35 mg/kg and declined by a maximal 50% at higher doses (posiphen ≥35 mg/kg, $p < 0.05$). Non-linear modeling to the standard sigmoid curve generated an ED_{50} of 19 mg/kg (Figure 7B), $p = 0.01$.

Drug action $A\beta_{40}$ and $A\beta_{42}$ levels in mouse cerebral cortex. A slight, but non-significant decline, in $A\beta_{40}$ levels was achieved by both phenserine doses (Figure 8). In contrast, posiphen 7.5 mg/kg significantly lowered $A\beta_{40}$ (control: 14.53 ± 0.42 pg $A\beta_{40}$/mg brain tissue vs. 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. By 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-dependency ($r = -0.12592$, $p < 0.399$, $n = 47$) (Figure 9). The correlation coefficients for

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posiphen treatment effects on $A\beta_{40}$ levels vs. $A\beta_{42}$ 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 (Minneapolis, MN) were used without additional validation, recognizing that the assays may detect other proteases with similar specificities. β-secretase activity (Figure 10) was significantly reduced in brain extracts from posiphen-treated animals administered 35 and 50 mg/kg (control: 199.12 ± 17.85 fluorescence units (FU) /mg brain tissue vs. 159.64 ± 6.31 FU/mg and 143.74 ± 7.97 FU/mg, $p < 0.05$, Dunnett). Further, 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. By contrast, there was no trend associated with either α- or γ-secretase activity and phenserine or posiphen treatment (Figures 11A and B). The β-secretase assay signal exceeded that for the highest standard amount used (2 μg 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 a control samples eliminated β-secretase activity (Figure 12A). In addition, substrate specificity for the β-secretase assay was assessed by performing the assay with the substrates for α-, β-, and γ-secretases (Figure 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. Whereas presently approved anticholinesterases and memantine, are primarily useful for symptomatic relief (Lahiri et al., 2003b), they appear to have 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. As 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 whether or not common molecular mechanisms link the two targets (Lahiri et al., 2003a; Lahiri et al., 1998; Lahiri et al., 1994; Shaw et al., 2001). This work culminated in the development of carbamates on the backbone of hexahydropyrrolo[2,3b]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 previously reported 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 (i) 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, (ii), cholinergic forebrain (nucleus basalis) lesions in rats that resulted

in a rapid, 1 hr, and sustained elevation of APP and Aβ in cortex and CSF (Haroutunian et al., 1997; Wallace et al., 1991). This latter model mimics the forebrain cholinergic loss found in AD. As 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 likely results from actions occurring prior to protein synthesis. Furthermore, the rapidity of the elevation eliminates transcriptional induction that has been estimated to require 4 hr for the APP gene. Hence, the increase in APP protein is likely 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-beta peptide levels (Utsuki et al., 2006).

The present studies support this concept demonstrating consistent posiphen and phenserine-induced concentration-dependent reductions of APP synthesis in neuronal cells in culture, as assessed by measuring steady-state levels after 16 h and 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 synthesis 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 0.67 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. Whereas actions on APP and Aβ translated from culture to *in vivo* for both compounds were similar at the same dose, phenserine-mediated

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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 ACh levels (Greig et al., 2005). By 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 phenserine 7.5 mg/kg: 13%, 7.5% and 33%; and for posiphen 7.5 mg/kg 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 TGF-β responsive element and an iron responsive element, all of which, in reaction to appropriate ligands, can up regulate APP levels (Lahiri et al.,

2003a; Rogers et al., 1999). 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 synaptic 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 (Klegeris and McGeer, 2005). Whether or not phenserine and posiphen, in addition to numerous proinflammatory cytokine that are elevated in AD brain, share these remains to be elucidated

Our results suggest that β-secretase activity can also be regulated by posiphen, as 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 (unpublished). 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 (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), 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 maximum reduction in Aβ levels achieved was approximately 50%, it is not known why the reduction leveled off around 50% from the control. However, in another set of animal experiments, the reduction in Aβ level was found to be small (~10%) but significant between posiphen vs. control (unpublished data). Such variation could be due either 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 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, need 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 appear 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, that 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.

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Legends for Figures:

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).

Fig. 2. Posiphen effects on APP and Aβ levels in human SK-N-SH cells. Concentration-dependent action of posiphen on (A) secreted and intracellular APP levels and (B) secreted Aβ levels in human neuroblastoma (SK-N-SH) cells in culture at 16 hr of treatment (mean $\pm$ SEM). (A) All levels reduced compared to controls, and (B) 16 hr levels at 50 μM reduced compared to 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.

Fig. 3. Concentration-dependent action of posiphen on APP levels in human SH-SY-5Y cells. Concentration-dependent action of A) phenserine (0.2-20 μM) and B) posiphen (0.2-20 μM), on secreted and intracellular APP levels in human neuroblastoma (SH-SY-5Y) cells in culture (mean $\pm$ SEM) as assessed at 16 hr. 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 $[S]^{35}$ incorporated protein levels (trichloroacetic acid (TCA) precipitable counts) normalized by non-precipitable counts, and was unaffected by posiphen and phenserine (10 μM). (C) Newly synthesized APP levels were then normalized by S^{35} 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 RT-PCR. Treatment with posiphen and phenserine did not affect APP mRNA levels ($p > 0.05$, Dunnett).

Fig. 5. Brain hemisphere mass and total protein per brain hemisphere. White bars indicate control (saline i.p.) mice. Stipple indicates phenserine-treated mice. Diagonal hatch indicates posiphen-treated mice. Error bars indicate standard error of the mean. 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 $p < 0.05$ indicated with asterisk. 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 $p < 0.05$ indicated with asterisk.

Fig. 6. Western blot of mouse brain extracts for APP and β-actin. Mouse brain extracts were run on SDS-PAGE, 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. Rest of the procedures for Western blotting is as

described in the text. APP band is indicated (left of blot), as are nearest molecular weight 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 weight marker positions (right of blot).

Fig. 7. Phenserine and posiphen treatment effects on APP levels. A. White bar indicates control (saline i.p.) mice. Stipple indicates phenserine-treated mice. Diagonal hatch indicates posiphen-treated mice. Error bars indicate standard error of the mean. APP was measured by western blot and adjusted for actin and tissue mass as indicated in the text. Samples significantly different from control at $p < 0.05$ indicated with asterisk. B. Dose-response model of posiphen activity on adjusted APP levels in mouse cerebral cortex hemispheres. Non-linear 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 indicate control (saline i.p.) mice. Stipple indicates phenserine-treated mice. Diagonal hatch indicates posiphen-treated mice. Error bars indicate standard error of the mean. 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 $p < 0.05$ indicated with asterisk. B. Relative $A\beta_{40}$ levels in mouse cerebral cortex (control = 1). Samples significantly different from control at $p < 0.05$ indicated with asterisk.

Fig. 9. Phenserine and posiphen dose effects on $A\beta_{42}$ levels in mouse cerebral cortex. White bars indicate control (saline i.p.) mice. Stipple indicates phenserine-treated mice. Diagonal hatch indicates posiphen-treated mice. Error bars indicate standard error of the mean. 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 $p < 0.05$ indicated with asterisk. B. Relative $A\beta_{42}$ levels in mouse cerebral cortex (control = 1). Samples significantly different from control at $p < 0.05$ indicated with asterisk.

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 indicate control (saline i.p.) mice. Diagonal hatch indicates posiphen-treated mice. Error bars indicate standard error of the mean. A. β-secretase activity in mouse cerebral cortex, on a fluorescence unit per mg brain tissue basis. Samples significantly different from control at $p < 0.05$ indicated with asterisk. B. β-secretase assay raw FU.

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 mg 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 mg brain tissue basis. White bars indicate control (saline i.p.) mice. Diagonal hatch indicates posiphen-treated mice. Error bars indicate standard error of the mean. 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 indicates activity for control samples. Diagonal hatch indicates activity for 50 mg/kg 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.

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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% of enzyme activity

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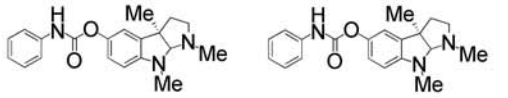
Figure 1



(−)–Phenserine (phenserine) (+)–Phenserine (posiphen)

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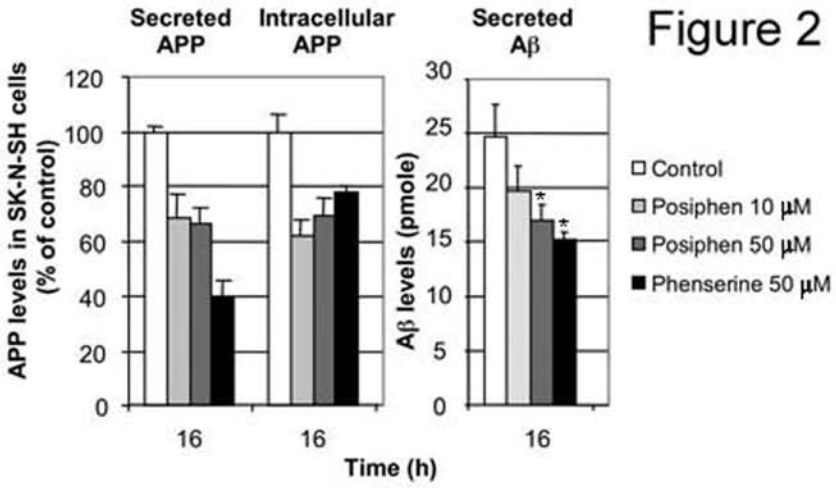

Figure 2

Figure 3.

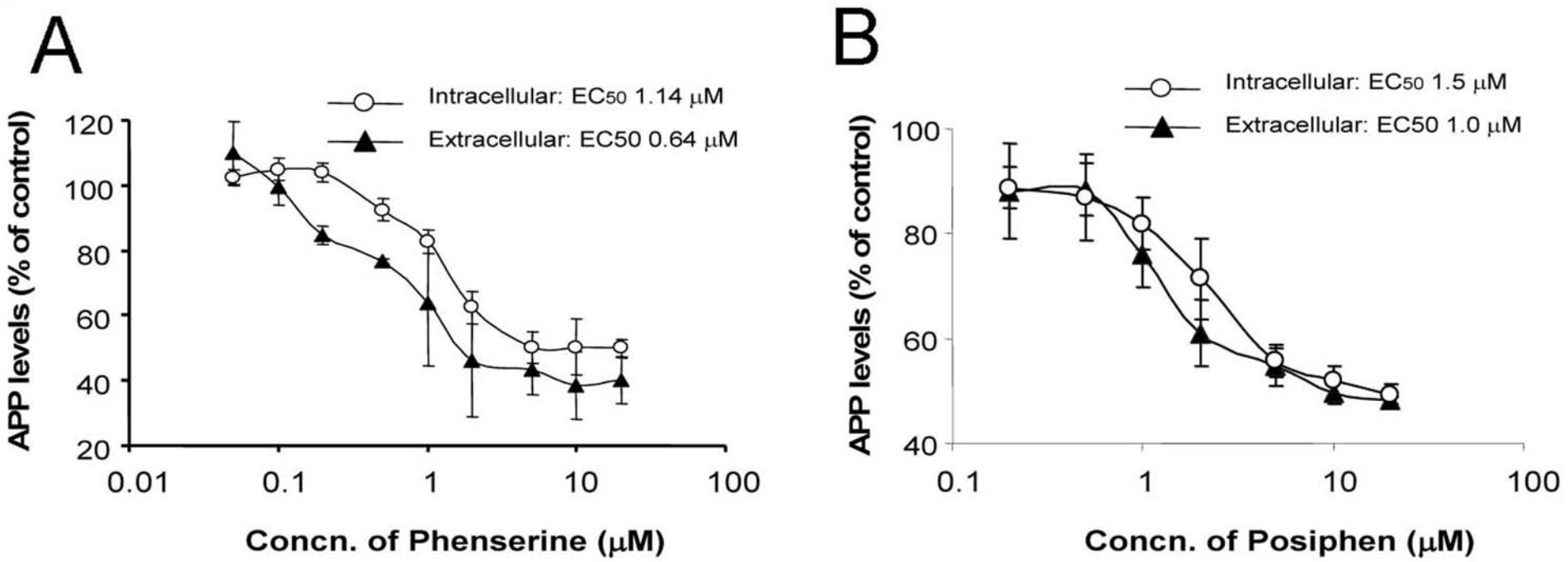

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Figure 4.

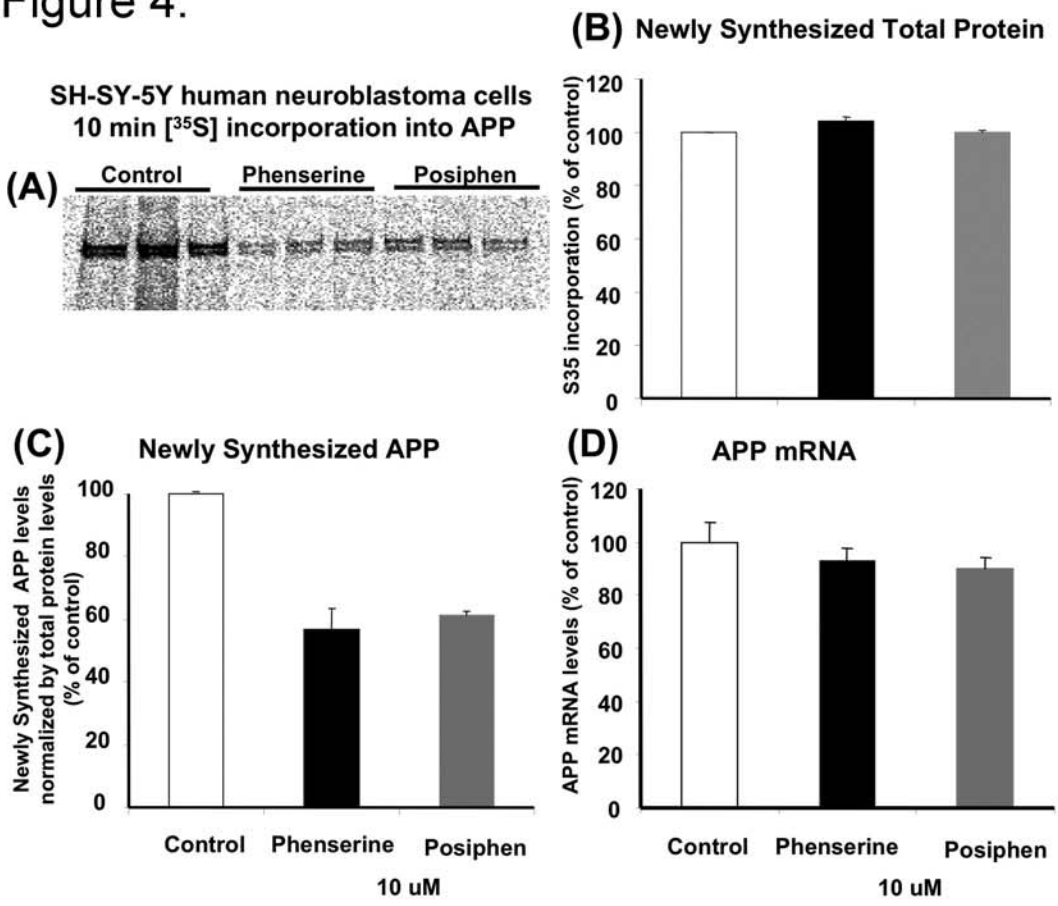

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Figure 5

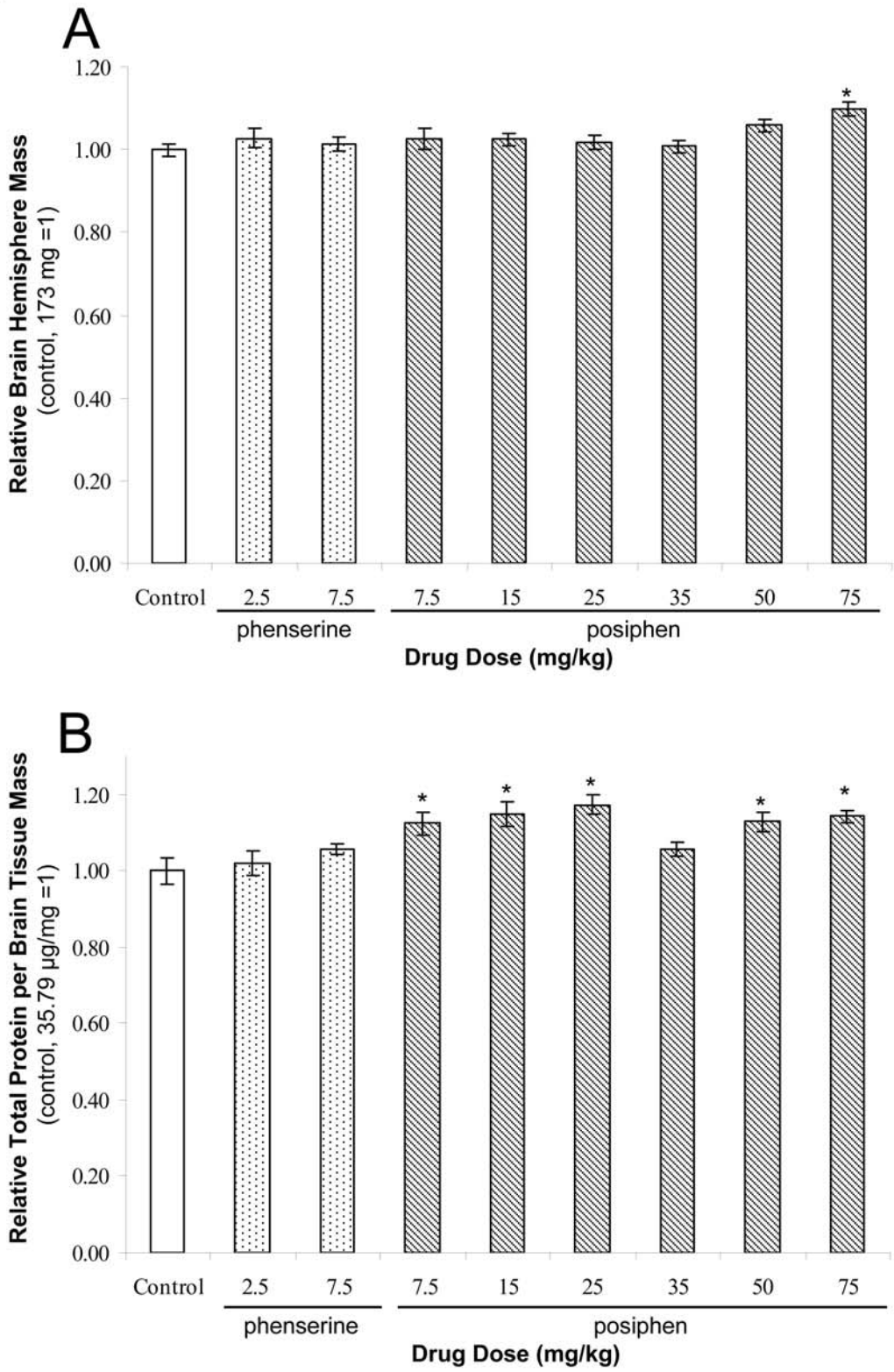

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Figure 6

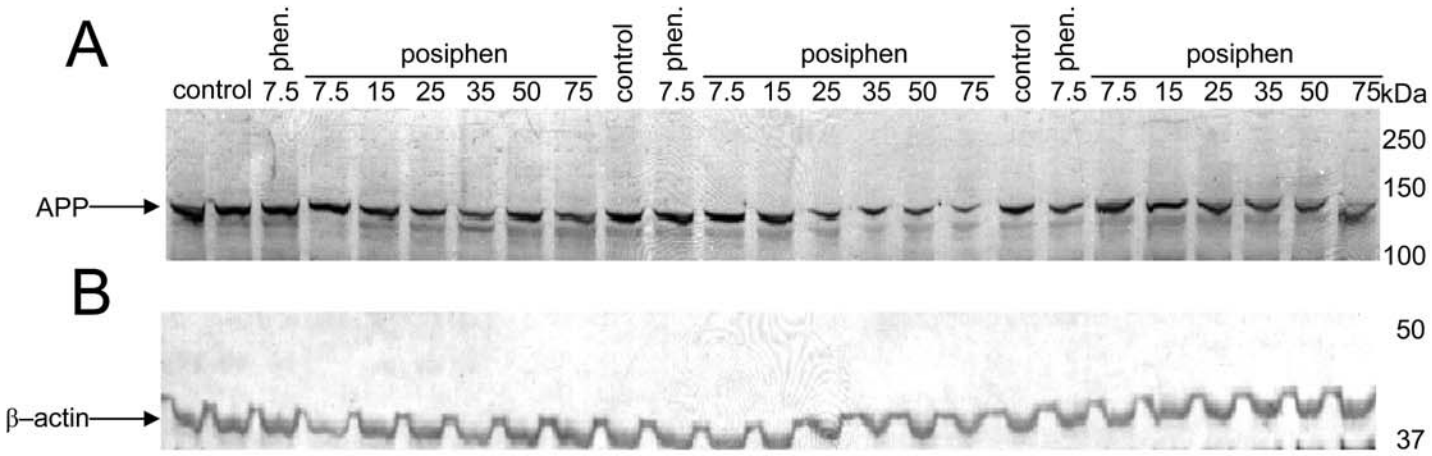

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Figure 7

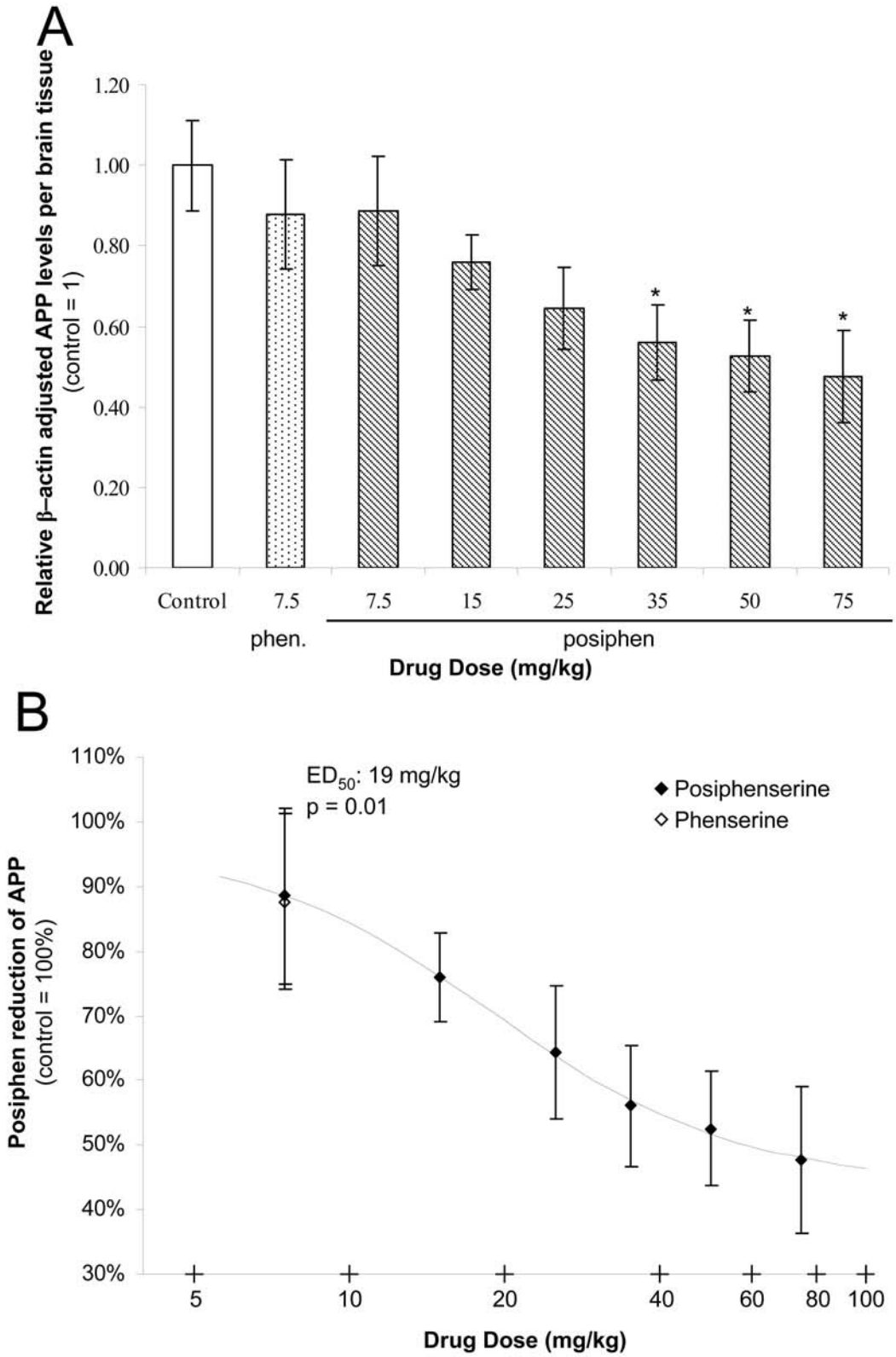

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Figure 8

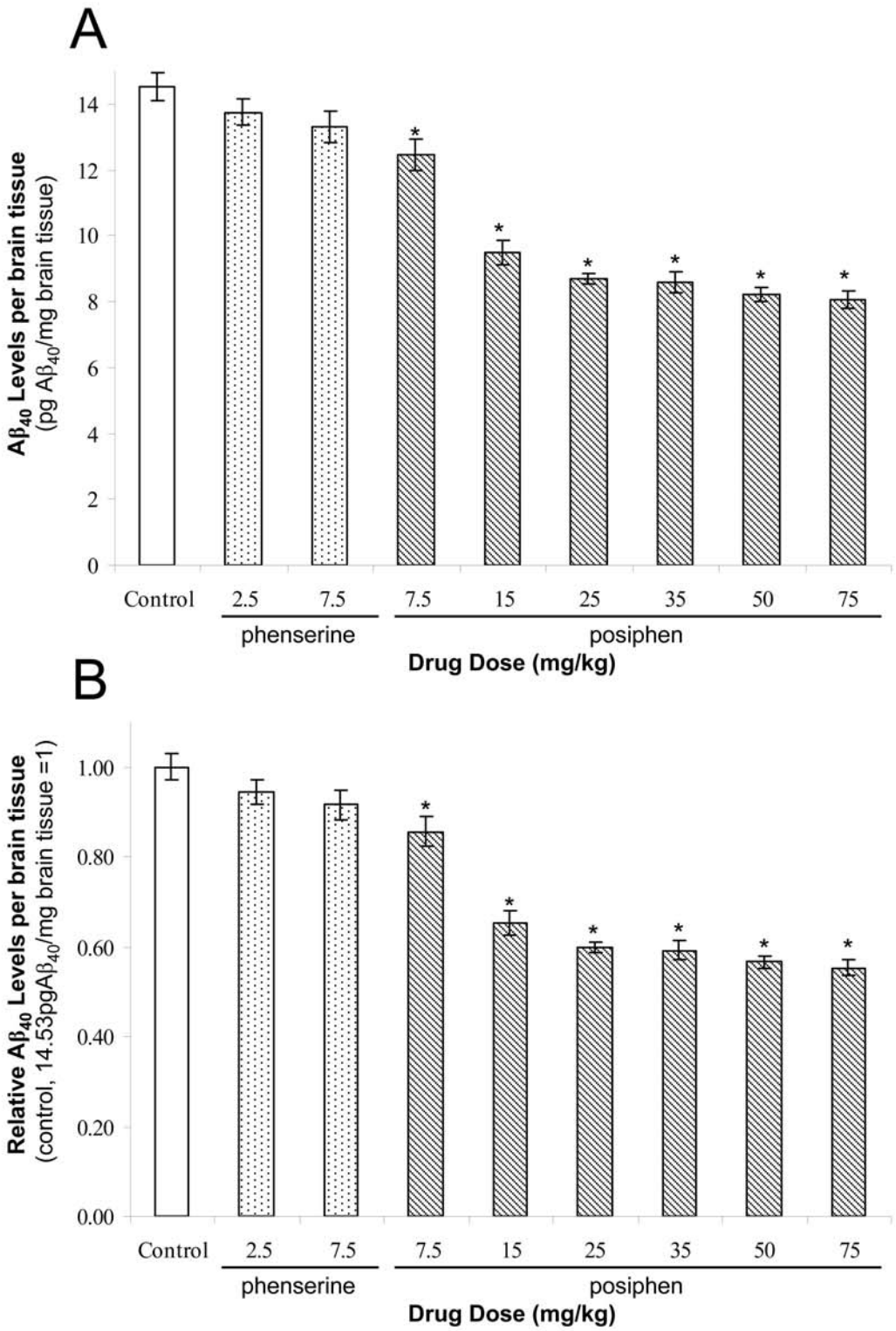

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Figure 9

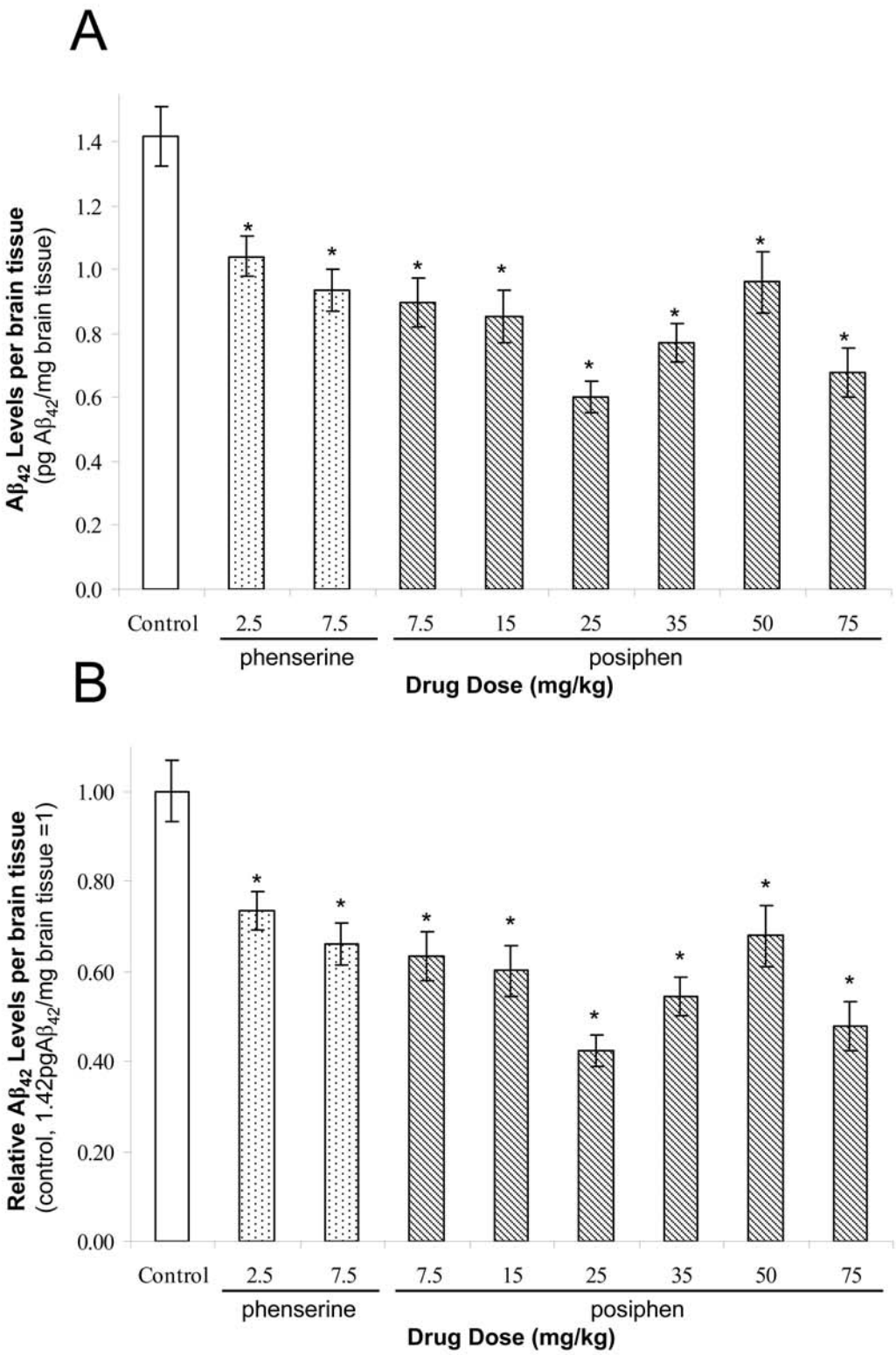

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Figure 10

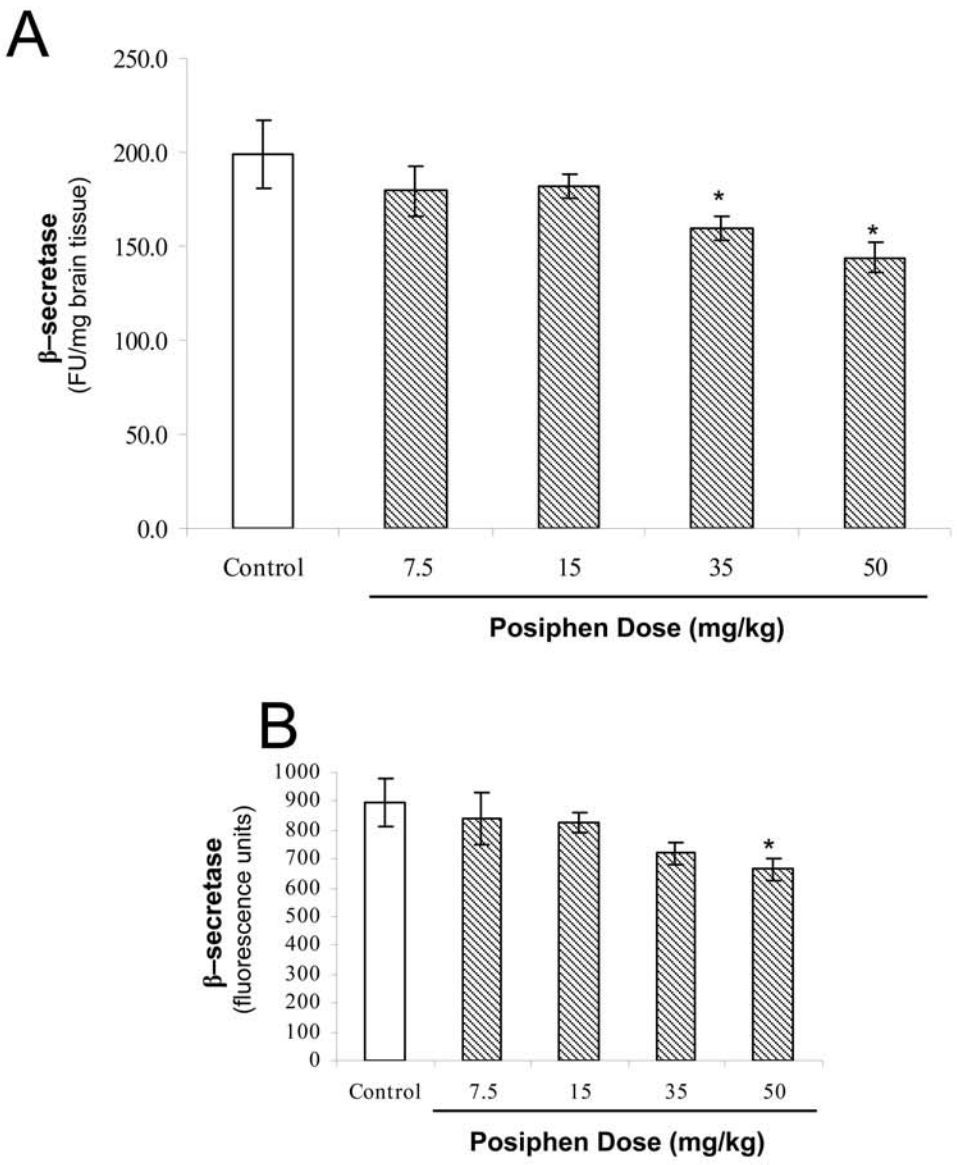

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Figure 11

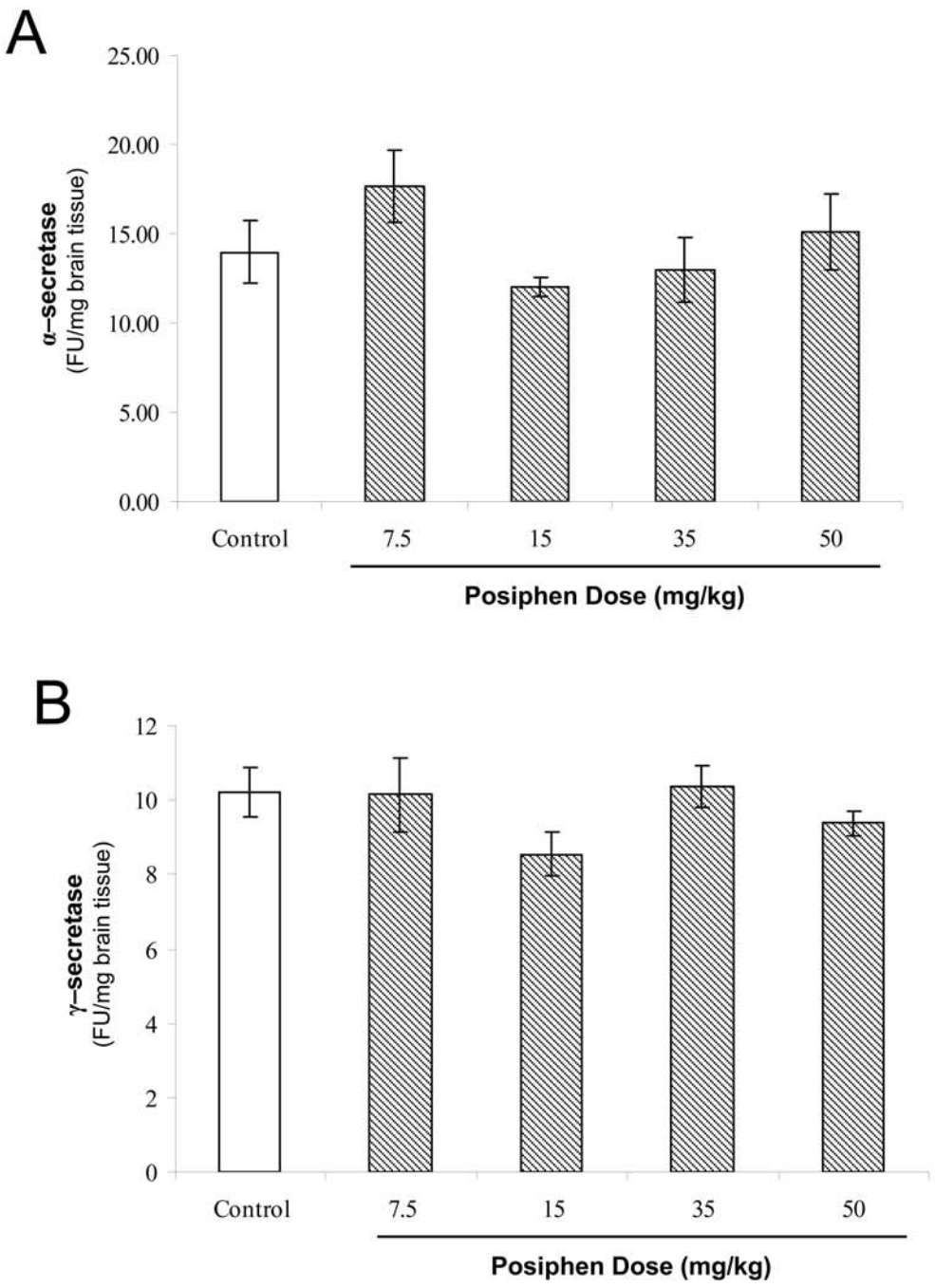

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Figure 12